Canadian Journal of Biochemistry and Physiology

Editor: J. B. COLLIP

Associate Editors:

L.-P. DUGAL, Laval University

J. K. W. FERGUSON, University of Toronto

C. S. HANES. University of Toronto

A. G. McCalla, University of Alberta

F. C. MACINTOSH, McGill University

R. J. ROSSITER, University of Western Ontario

Canadian Journal of Biochemistry and Physiology

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY and five other journals devoted to the publication, in English or French, of the results of original scientific research. La Revue accepte des travaux originaux on biochimie, physiologie, pharmacologie, et sujets connexes.

Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies. The Canadian Biochemical Society, the Canadian Physiological Society, and the Pharmacological Society of Canada have chosen the Canadian Journal of Biochemistry and Physiology as their official journal for the publication of scientific papers.

EDITORIAL BOARD

Representatives of the National Research Council

- I. McT. Cowan (Chairman), University of British Columbia LEO MARION, National Research Council
- A. D. MISENER, Ontario Research Foundation D. L. THOMSON, McGill University

Editors of the Journals

- D. L. Bailey, University of Toronto T. W. M. Cameron, Macdonald College F. E. Chase, Ontario Agricultural College H. E. Duckworth, McMaster University
- LEO MARION, National Research Council J. F. MORGAN, Department of National Health and Welfare, Ottawa J. A. F. STEVENSON, University of

Representatives of Societies

- D. L. BAILEY, University of Toronto
- Royal Society of Canada

 T. W. M. CAMERON, Macdonald College
 Royal Society of Canada

 H. E. DUCKWORTH, McMaster University
 Royal Society of Canada

 Canadian Association of Physicists
- P. R. GENDRON, University of Ottawa Chemical Institute of Canada

Western Ontario

- D. J. LE ROY, University of Toronto
 Royal Society of Canada
 J. F. MORGAN, Department of National Health
 and Welfare, Ottowa
 Canadian Biochemical Society
 R. G. E. MURRAY, University of Western Ontario
 Canadian Society of Microbiologists
 J. A. F. STEVENSON, University of
 Western Outgoin
- Western Ontario Canadian Physiological Society

Ex officio

LÉO MARION (Editor-in-Chief), National Research Council J. B. MARSHALL, Division of Administration and Awards, National Research Council

Manuscripts for publication should be submitted to Dr. J. F. Morgan, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa (Biochemistry), or to Dr. J. A. F. Stevenson, Head, Department of Physiology, University of Western Ontario, London, Ontario (Physiology and Pharmacology).

For instructions on preparation of copy, see NOTES TO CONTRIBUTORS (inside back cover).

Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration and Awards, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration and Awards, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and subscription prices are:

Canadian Journal of Biochemistry and Physiology	Monthly	. \$ 9.00 a year
Canadian Journal of Botany	Monthly	\$ 6.00
Canadian Journal of Chemistry	Monthly	\$12.00
Canadian Journal of Microbiology	Bimonthly	\$ 6.00
Canadian Journal of Physics	Monthly	\$ 9.00
Canadian Journal of Zoology	Bimonthly	\$ 5.00

The price of regular single numbers of all journals is \$2.00.





CORRECTION

Can. J. Biochemistry and Physiology. Vol. 33, p. 888. The following tabular material should be substituted below the heading of Table I.

Day of study	8 a.m.	12 noon	3.30 p.m.
11	.63	.61	_
12*	.51	.26	.50
13	.43	.33	.40
14	.47	.48	_

^{*}Infusion of aldosterone.



Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 34

MARCH 1956

NUMBER 2

THE METABOLISM OF THE ERYTHROCYTE

X. THE INORGANIC PYROPHOSPHATASE OF THE ERYTHROCYTE

By A. Malkin² and O. F. Denstedt

Abstract

The activity of the pyrophosphatase which catalyzes the hydrolysis of inorganic pyrophosphate in the crythrocyte of the human, the rabbit, and the chicken is confined entirely to the cytoplasm of the cell. Following preincubation, the enzyme activity in the human crythrocyte is diminished, but preincubation in the presence of cysteine or glutathione prevents the diminution of the enzyme activity. Aging of the hemolyzate of the human crythrocytes results in a marked loss of the inorganic pyrophosphatase activity. The diminished activity can be restored by the addition of cysteine or glutathione to the reaction mixture; but after the hemolyzate has aged for five or six days at 5°C. the loss in the enzyme activity can no longer be restored with these at 5° C., the loss in the enzyme activity can no longer be restored with these reagents. Fluoride and calcium ions inhibit the activity of the enzyme, while magnesium ions are essential for its activity. Calcium is a noncompetitive inhibitor, while the inhibition by fluoride is of a "quadratic" nature. If a constant ratio of magnesium to pyrophosphate is maintained, the quadratic inhibition can be converted to the "uncompetitive" type of inhibition.

Introduction

Several biochemical reactions have recently been discovered in which inorganic pyrophosphate is a by-product of the reaction. Five of these are discussed below.

1. Kornberg (13) isolated, in a purified form, an enzyme from autolyzates of ale yeast and also from extracts of an "acetone-dried" powder from hog liver which was found to catalyze the following reaction:

2. Lipmann et al. (10) demonstrated the synthesis of acetyl CoA by purified enzyme preparations from pigeon liver and yeast and found pyrophosphate to be among the products of the over-all reaction, as follows:

ATP + acetate + CoA = AMP + PP + acetyl CoA.

¹Manuscript received September 30, 1955.

Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec. This work was supported by the Defence Research Board of Canada, Grant number 9350-01, Project

²J. B. Collip Fellow in Medical Research, McGill University, 1953-54. Medical Research

Fellow, National Research Council, Canada, 1954-56.

*The following abbreviations are used throughout this paper: NMN, nicotinamide mononucleotide; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; PP, inorganic pyrophosphate; CoA, coenzyme A; UTP, uridine triphosphate; UDPG, uridine diphosphate glucose; AMP, adenosine monophosphate; PRPP, 5'-phosphoribosylpyrophosphate.

3. Kalckar (11) showed that the synthesis of UDPG proceeded in the following manner:

4. The preliminary stages of the synthesis of protein by rat liver was shown by Hoagland (8) to occur in much the same way as the synthesis of acetyl CoA (reaction 2). The sum of the series of reactions may be written as follows, with hydroxylamine as the amino acid acceptor:

5. Kornberg et al. (14) have recently purified an enzyme from yeast which catalyzes the synthesis of AMP in the following way:

The same enzyme appears to be capable of catalyzing the synthesis of inosinic acid and guanylic acid from hypoxanthine and guanine, respectively; while a different enzyme, purified from yeast as well, can catalyze the synthesis of orotidine-5'-phosphate from orotic acid (15). The mechanism for the synthesis of this compound is the same as for the synthesis of AMP.

The first three and the last reactions shown here are completely reversible, while in the fourth, the intermediate steps leading to the formation of the hydroxamic acid – amino acid (not illustrated) are all reversible, only the final stage of the reaction being irreversible.

The accumulation of inorganic pyrophosphate in any cell in which any of the foregoing reactions can occur would tend to shift the equilibrium of the reaction to the left. The removal of inorganic pyrophosphate appears to be ensured, however, by the presence of the enzyme, inorganic pyrophosphatase, which catalyzes the hydrolysis of the inorganic pyrophosphate. It has been found in a wide variety of plant and animal tissues (12, 19, 7, 1, 24, 20, 17, 6). In all instances, magnesium was found to be required for the activation of the enzyme, although the activation appears to occur by virtue of the formation of a magnesium-pyrophosphate complex prior to the hydrolysis of the substrate (24, 25, 2). Liver, brain, yeast, and erythrocyte pyrophosphatases appear to require free sulphydryl groups for optimum activity (24, 7, 1, 20), although the enzyme when partially purified from extracts of firefly 'lanterns' was not activated by cysteine, nor was it inhibited by alloxan or iodoacetate (17). Calcium and fluoride were found to inhibit the enzyme, but only Bailey et al. (1) studied the nature of the inhibition by calcium. workers showed that yeast pyrophosphatase was inhibited by calcium, the inhibition being noncompetitive. Apparently, no kinetic study has been done to ascertain the nature of the inhibition of pyrophosphatase by fluoride. Swanson (24), however, has pointed out that the inhibition by fluoride is due to the formation of a magnesium-fluoro-pyrophosphate complex, which competes with magnesium pyrophosphate, the true substrate, for the enzyme.

Because of the apparent importance of this enzyme in certain biosynthetic mechanisms, a further study of some of its characteristics was warranted.

Material and Methods

In the studies on inorganic pyrophosphatase in the red blood cell, human blood was used in most of the experiments. Blood was collected in heparin, centrifuged, and the plasma and 'buffy layer' removed. The red blood cells were washed three times with 0.9% sodium chloride, then diluted to 10 times the sedimented volume with saline. The erythrocytes were then hemolyzed by freezing (in a dry-ice – alcohol mixture), then thawing (in a water bath kept at 37° C.). This procedure was repeated twice. The hemolyzate was dialyzed against running tap water for two to four hours, then stored at 5° C. until required for the experiment. To ascertain the distribution of the enzyme within the erythrocyte, the hemolyzate was centrifuged at 3000 r.p.m., and the stroma-free supernatant (SFH) was removed. The stroma residue was washed five or six times with saline, and made up to the volume of the original hemolyzate.

For the studies on the distribution of the pyrophosphatase within the nucleated erythrocyte, chicken blood was collected in heparin, centrifuged, hemolyzed, and fractionated into the SFH and particulate material as described above.

Similar studies were carried out with the rabbit reticulocytes and erythrocytes. The reticulocytes were obtained from animals with an induced reticulocytosis (50%). Blood was collected in heparin, the reticulocytes were washed, hemolyzed, and fractionated into SFH and particulate material as previously described.

The erythrocytes were obtained from normal rabbits and fractionated. The pyrophosphatase activity was compared with that of the reticulocyte

preparations.

Inorganic pyrophosphatase activity was determined by measurement of the amount of inorganic phosphate liberated during the incubation of sodium pyrophosphate in the presence of the enzyme and magnesium chloride. The optimal concentration of magnesium chloride was found to be 0.02 M. The inorganic phosphate was measured by the method of Fiske and SubbaRow (5).

Results

Maizels et al. (3) in a study on the distribution of phosphatases in human erythrocytes observed that the inorganic pyrophosphatase activity was confined to the SFH and that no activity was demonstrable in the stroma. Swanson (24) showed that in rat liver homogenates, the pyrophosphatase activity is concentrated in the soluble portion. In the human erythrocyte, the rabbit reticulocyte, and the chicken erythrocyte, the pyrophosphatase activity is confined entirely to the soluble or cytoplasmic fraction of the cells (Table I). The activity in the rabbit reticulocyte was found to be about 15 times as great as that of the erythrocyte.

The pyrophosphatase activity of specimens of human erythrocytes falls off significantly within a few days (at 5°C.) following collection, but the activity in the aliquots used for the assay can be restored by the addition of

TABLE I

INORGANIC PYROPHOSPHATASE ACTIVITY OF THE RABBIT RETICULOCYTE AND THE RABBIT, HUMAN, AND CHICKEN ERYTHROCYTE

Fraction	Phosphorus liberated (µM./cc. fraction/hr.)	F	lecovery %
Rabbit erythrocyte hemolyzate	30		
Rabbit reticulocyte hemolyzate	444		***
Rabbit reticulocyte SFH Rabbit reticulocyte particulate fraction	347 2.9		79
		Total	80
Human erythrocyte hemolyzate	212		
Human erythrocyte SFH	214		101
Human erythrocyte particulate fraction	5.5		2.5
		Total	103.5
Chicken erythrocyte hemolyzate	201		
Chicken erythrocyte SFH	158		78
Chicken erythrocyte particulate fraction	3.3		2
		Total	80

TABLE II

THE EFFECT OF CYSTEINE AND GLUTATHIONE ON THE INORGANIC PYROPHOSPHATASE ACTIVITY OF THE HUMAN ERYTHROCYTE

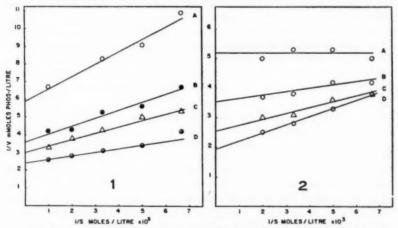
Day of collection	Preincubation	Cysteine	Glutathione	Phosphorus liberated (mM./liter)
Day 0	-	_	_	0.34
Day o	_	+	_	0.39
	_	_	+	0.35
	+	_	_	0.14
	+	+	-	0.48
	+	_	+	0.45
Day 3	-	_	-	0.14
	_	+	****	0.26
	_	_	+	0.23
	+	_	_	0.07
	+	+	-	0.27
	+	_	+	0.13
Day 6	_	_	_	0
	_	+	-	0.07
	-	_	+	0.01
	+	-	-	0
	+	+	-	0.04
	+	-	+	0

Incubation medium consisted of: 0.01 M PP, 0.2 ml.; 0.4 M MgCl₂, 0.1 ml.; 0.2 M cysteine, 0.1 ml.; 0.2 M glutathione, 0.1 ml.; erythrocyte hemolyzate, 0.1 ml.; 0.1 M Tris buffer pH 7.5, to 2.0 ml. Preincubation for 10 min. at 37° C. where indicated. Incubation 15 min. at 37° C. Precipitation with 8.0 ml. of 10% trichloracetic acid. Inorganic phosphate determined on aliquots of the filtrate.

cysteine or glutathione to the reaction mixture (Table II). Similarly, preincubation of the reaction mixture resulted in a diminution of the enzyme activity, while the addition of cysteine or glutathione beforehand was found not only to protect the enzyme, but restore the activity to normal or further increase it. By the fifth or sixth day the inorganic pyrophosphatase activity falls almost to zero, and the addition of either cysteine or glutathione is no longer effective in restoring or increasing the activity.

Calcium ions inhibit the pyrophosphatase activity of the human erythrocyte, the inhibition being of the noncompetitive type (Fig. 1). The value for K_i was found to be $4 \times 10^{-4} M$, and for K_m , $1.2 \times 10^{-4} M$. In this experiment, the pyrophosphate concentration was varied, while the magnesium concentration was kept constant at a final concentration of 0.02 M. When the magnesium ion concentration was varied along with that of the inorganic pyrophosphate, while the ratio of the concentrations of magnesium to pyrophosphate was kept constant at 20:1, the inhibition of the enzyme by calcium ions still was of a noncompetitive character.

Swanson (24) has suggested that calcium ions inhibit the inorganic pyrophosphatase of liver by forming a calcium-pyrophosphate complex which competes with magnesium pyrophosphate for the enzyme. If this were so, one would expect to find a competitive rather than noncompetitive type of inhibition of the enzyme by calcium ions. There are at least two possible explanations for the discrepancy between the hypothetical case and our observations. In the first instance, magnesium pyrophosphate may not be the "true substrate" for the enzyme, magnesium acting merely as a metallic



FIGS. 1 and 2. The effect of calcium chloride and of sodium fluoride on the inorganic pyrophosphatase activity of the human erythrocyte. All the concentrations are expressed as the final concentrations.

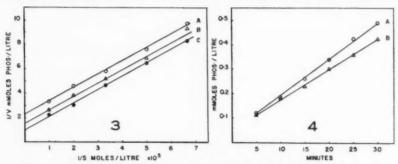
Fig. 1. A. Calcium chloride, 5×10^{-4} M. B. Calcium chloride, 2×10^{-4} M. C. Calcium chloride, 1×10^{-4} M. D. No calcium chloride added.

Fig. 2. A. Sodium fluoride, 5×10^{-5} M. B. Sodium fluoride, 2.5×10^{-5} M. C. Sodium fluoride, 1×10^{-5} M. D. No sodium fluoride added.

activator, with inorganic pyrophosphate as the substrate. If this were so, we should expect competition between the calcium and the magnesium ions for an active site on the enzyme surface, and this, in effect, would constitute a competitive inhibition. This, however, does not occur. The alternative possibility is that magnesium pyrophosphate is the 'true substrate' for inorganic pyrophosphatase. Calcium ions, instead of competing with this complex, appear to render inactive a particular part of the enzyme surface, the over-all effect being one of noncompetitive inhibition, such as we have observed. From our observations on the effect of calcium ions on inorganic pyrophosphatase, it would appear, as Bloch-Frankenthal has suggested (2), that magnesium pyrophosphate is, indeed, the true substrate for the inorganic pyrophosphatase of the human erythrocyte. In other words, the metallic activator appears, in this case, to be part of a metallo-substrate complex.

The nature of the quadratic type of inhibition is somewhat obscure. Ebersole *et al.* (4) suggest that in many biological processes, the enzyme-catalyzed reaction is not a simple one capable of formulation by the Michaelis-Menten scheme, and that the enzyme-substrate complex undergoes dissociation in several steps. They postulate that one of the products which ordinarily would be involved eventually in the reaction, instead combines with the inhibitor. According to Ebersole *et al.* (4) this rather unusual type of inhibition can be represented in the following way. When the reciprocal of the substrate concentration is plotted against the reciprocal of the velocity, the straight lines obtained for various values of the inhibitor have different intercepts on the axis. They begin with a slope of nearly zero, then change to a common slope equal to that obtained when the concentration of the inhibitor is zero. This effect is clearly evident in the figure (Fig. 2) in which the lines diverge towards the ordinate.

This type of inhibition may be related to another uncommon variety, namely, "uncompetitive" inhibition. In the experiment discussed in the



FIGS. 3 and 4. The effect of fluoride on the inorganic pyrophosphatase activity of the human erythrocyte. All the concentrations are expressed as the final concentrations. FIG. 3. The concentration of magnesium was varied along with that of sodium pyrophosphate, so that the magnesium to pyrophosphate ratio was always 20:1. A. Sodium fluoride, $5 \times 10^{-5} M$. B. Sodium fluoride, $2.5 \times 10^{-5} M$. C. No fluoride added.

Fig. 4. A. No sodium fluoride. B. Sodium fluoride, $1 \times 10^{-5} M$.

foregoing paragraph, the inorganic pyrophosphate concentration was varied while the magnesium concentration was maintained constant at 0.02 M, that is, at a level which was found to be the optimal concentration for the maximum amount of pyrophosphate used. In the following experiment, the magnesium concentration was varied along with that of pyrophosphate, so that the magnesium to pyrophosphate ratio was always 20:1. Sodium fluoride was used as the inhibitor. In this instance, we observed that the inhibition of the enzyme by sodium fluoride was of the uncompetitive type (Fig. 3). In other words, if the reciprocal of the substrate concentration be plotted against the reciprocal of the velocity of the reaction (16), the intercept increases with increasing concentration of sodium fluoride, but the slope remains unchanged. This type of inhibition arises from the circumstance that the inhibitor can combine with the enzyme-substrate complex, but not with the enzyme alone (4). Since the K_* (Michaelis constant) is equal to the slope divided by the intercept (when plotted according to the method of Lineweaver and Burk (14)), in uncompetitive inhibition, the apparent K_s diminishes (the intercept increases but the slope remains unchanged). Thus, with an increase in the concentration of the inhibitor, the enzyme-substrate dissociation constant is diminished or the affinity of the enzyme for the substrate is increased. quadratic inhibition, we observed that a nearly zero slope was obtained with the limiting concentrations of the inhibitor (Fig. 2). In such cases, then, the enzyme-substrate dissociation constant approaches zero, and the affinity of the enzyme for the substrate approaches infinity.

Further evidence suggesting that there may be a relationship between these two rather uncommon forms of inhibition is apparent from the figure showing quadratic inhibition (Fig. 2). It may be seen that with the decrease in the inhibitor concentration there is a tendency for the line to become parallel to that observed in the absence of the inhibitor (uncompetitive inhibition). Thus it would seem possible that the quadratic and uncompetitive types of inhibition are but different manifestations of the same type of inhibition, produced by varying the concentration of either the activator (such as Mg⁺⁺) or of the inhibitor.

Quadratic inhibition of phosphoglucomutase (21), enolase (25), and lecithinase (22) has been observed. In all of these cases, sodium fluoride was the inhibitor, and magnesium was the metallic activator. With lecithinase, either calcium or magnesium ions can serve equally well as activator. In the case of phosphoglucomutase, Najjar (21) considered that the "true inhibitor" is actually a magnesium–fluoro–glucose-1-phosphate or a magnesium–fluoro-glucose-6-phosphate complex. For enolase, Warburg (25) suggested that the inhibitor was a magnesium–fluoro–phosphate complex. With lecithinase, Najjar (22) considered that the true inhibitor of this enzyme was a magnesium–fluoro–lecithin complex. In the case of the inorganic pyrophosphatase, the inhibitor appears to be a magnesium–fluoro–phosphate complex, rather than a magnesium–fluoro–substrate complex. When the enzyme activity was followed over a period of time, both in the presence and in the absence of

sodium fluoride, a progressive inhibition was observed with fluoride as the reaction proceeded (Fig. 4). This could mean that with the progressive hydrolysis of the substrate, there was an increasing formation of the inhibitor complex whose accumulation tends to retard the reaction. In the case of inorganic pyrophosphatase, therefore, the true inhibitor, in the presence of fluoride, appears to be a magnesium-fluoro-phosphate complex; that is, a complex of the product of the reaction, rather than of the substrate.

Discussion

One of the difficulties encountered in the study of the quadratic inhibition is that the concentration of the true inhibitor is not known, nor can it be determined. The concentration changes continuously, whether it is a complex of the substrate or of the product. Nevertheless, from a graphical analysis of the data the type of inhibition becomes evident. The problem then is to determine the nature of the inhibitor complex, and the manner by which this complex affects the enzyme-catalyzed reaction.

In the quadratic type of inhibition, the true inhibitor apparently may be a complex of the substrate or of the product depending on the enzyme system in question. With inorganic pyrophosphatase, the true inhibitor appears to be a complex of the product of the reaction. In the lecithinase system (22), the inhibitor is evidently a substrate complex, while with phosphoglucomutase (22) the inhibitor may be either a substrate complex or a complex of the product. With a given enzyme system, and under a given set of conditions, quadratic inhibition appears to be produced by the formation of an inhibitor complex, part of this complex representing either the substrate, the product,

or, as in the case of enolase, simply inorganic phosphate.

With respect to the manner by which the inhibitor complex exerts its effect, Najjar (22) pointed out that with the lecithin-lecithinase system, in the presence of fluoride, a magnesium-fluoro-lecithin complex is formed. The fluoride ions, apparently, have an affinity for the "active" sites on the lecithin molecule. In the form of the inhibitor complex, lecithin cannot undergo cleavage and thus the enzyme-catalyzed reaction is inhibited. The inhibitor complex may, of course, exert its effect in some other way. In our experiments on the inorganic pyrophosphatase of the human erythrocyte, the quadratic inhibition could be made to assume the characteristics of the uncompetitive type, apparently, by simply altering the concentration of the metallic activator, namely Mg++. We have pointed out, furthermore, that there may be a relation between quadratic and uncompetitive inhibition. Thus it may be that in quadratic inhibition, the inhibitor complex may exert its effect on the enzyme-substrate complex, as in uncompetitive inhibition, but not on the enzyme alone. The observed difference between the two types of inhibition could be due to the difference in concentrations of a metallic activator or an inhibitor, such as sodium fluoride, since these substances form part of the inhibitor complex.

It is important to note that in all the aforementioned examples of quadratic inhibition, the metallic activator forms part of the inhibitor complex. That

simple removal of magnesium would produce inhibition to the extent observed in these enzyme systems is hardly likely since with inorganic pyrophosphatase, the amount of magnesium present is so large compared with that of fluoride and phosphate that the small amount that might be removed by complex

formation would not appreciably alter the rate of the reaction.

The importance of inorganic pyrophosphatase for cellular function has been alluded to in the introduction to this paper. We have noted that in the avian and mammalian erythrocyte, the enzyme is confined to the cytoplasmic fraction of the cell. DPN appears to be synthesized in the nuclei of mouse liver (9), and in the nuclei of the chicken erythroycte (18). Other biosynthetic mechanisms may occur in other areas of the cell. If inorganic pyrophosphate is liberated as a by-product of these reactions, its removal must be facilitated so that the equilibrium of the synthesizing process will not be disturbed. The removal of inorganic pyrophosphate is ensured by the presence of inorganic pyrophosphatase in the cytoplasm of the cell. In our zeal to determine precisely, within the cell, the location of particular biologic mechanisms, one must not forget the interrelationship of the various cellular components. The synthesis of DPN, for example, takes place in the nucleus of the chicken erythrocyte, while the disposal of one of the by-products of the synthesis, namely, inorganic pyrophosphate, takes place in the cytoplasm of the cell.

Thus the avian erythrocyte affords an example of a phenomenon which undoubtedly occurs in all somatic cells but which is very difficult to demonstrate, namely, the functional interrelationship between the nucleus and the

cytoplasm.

References

BAILEY, K. and WEBB, E. C. Biochem. J. 38: 394. 1944.
 BLOCH-FRANKENTHAL, L. Biochem. J. 57: 87. 1954.
 CLARKSON, E. M. and MAIZELS, M. J. Physiol. 116: 112. 1952.
 EBERSOLE, E. R., GUTTENTAG, G., and WILSON, P. W. Arch. Biochem. 3: 399. 1943.
 FISKE, C. H. and SUBBAROW, Y. J. Biol. Chem. 66: 375. 1925.
 GILMOUR, D. and CALABY, J. H. Enzymologia, 16: 34. 1953.
 GORDON, J. J. Nature, 164: 579. 1949.
 HOAGLAND, M. B. Federation Proc. 14: 73. 1955.
 HOGEBOOM, G. H. and SCHNEIDER, W. C. J. Biol. Chem. 197: 611. 1952.
 JONES, M. E., BLACK, S., FLYNN, R. M., and LIPMANN, F. Biochim. et Biophys. Acta, 12: 141. 1953.

- Jones, M. E., Black, S., Flynn, R. M., and Lipmann, F. Biochim. et Biophys. Acta, 12:141. 1953.
 Kalckar, H. M. Biochim. et Biophys. Acta, 12:250. 1953.
 Kay, H. D. Biochem. J. 22:1446. 1928.
 Kornberg, A. J. Biol. Chem. 182:779. 1950.
 Kornberg, A., Lieberman, I., and Simms, E. S. J. Biol. Chem. 215:417. 1955.
 Lieberman, I., Kornberg, A., and Simms, E. S. J. Biol. Chem. 215:407. 1955.
 Lineweaver, H. and Burk, D. J. J. Am. Chem. Soc. 56:658. 1934.
 McElroy, W. D., Coulombre, J., and Hays, R. Arch. Biochem. and Biophys. 32:207. 1951. 1951.

 MALKIN, A. and DENSTEDT, O. F. Can. J. Biochem. Physiol. 34: 130. 1956.
 NAGANNA, B. and MENON, V. K. N. J. Biol. Chem. 174: 501. 1948.
 NAGANNA, B., RAMAN, A., VENUGOPAL, B., and SRIPATHI, C. E. Biochem. J. 60: 215. 1955

NAJJAR, V. A. J. Biol. Chem. 175: 281. 1948.
 NAJJAR, V. A. Phosphorus metabolism. Vol. 1. Edited by McElroy, W. D. and Glass, B. The Johns Hopkins Press, Baltimore, Md. 1951. p. 500.
 ROBBINS, E. A., STILLBERG, M. P., and BOYER, P. D. Arch. Biochem. and Biophys. 54: 215. 1955.
 SWANSON, M. A. J. Biol. Chem. 194: 685. 1952.
 WARBURG, O. and CHRISTIAN, W. Biochem. Z. 310: 384. 1941-42.

THE METABOLISM OF THE ERYTHROCYTE

XI. SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN THE ERYTHROCYTE¹

By A. Malkin² and O. F. Denstedt

Abstract

DPN pyrophosphorylase activity has been demonstrated in chicken erythrocytes, but could not be found in rabbit reticulocytes nor in human or rabbit erythrocytes. In the erythrocyte of the chicken, the activity was confined to the particulate fraction, which consists mainly of cell nuclei. The implications of these findings with respect to the maturation of the erythrocyte and the metabolic interrelationship between the nucleus and the cytoplasm are discussed.

Introduction

The mechanism of the biosynthesis of DPN³ was clarified to some extent by Kornberg's (11) demonstration that an enzyme, purified from autolyzates of ale yeast and from extracts of an "acetone-dried" powder from hog liver, could catalyze the synthesis of DPN from the substrates NMN and ATP, as follows:

[1] $NMN + ATP \rightleftharpoons DPN + PP$.

Later, Hogeboom and Schneider (7) showed that in mouse liver homogenates, the enzyme activity is confined almost entirely to the nuclei. More recently, Baltus (2) further elucidated the intracellular distribution of this enzyme by demonstrating its presence in the nucleoli of starfish oöcytes. The enzyme catalyzing the above reaction is referred to as a pyrophosphorylase (22), but more specifically. DPN pyrophosphorylase.

In the course of a comprehensive study in our laboratory on the metabolism of the erythrocyte, it was of importance to investigate the biosynthesis of DPN in the red cell for the following reasons. First, Pappius *et al.* (18) previously had shown that during the preservation of human blood in the cold, DPN in the erythrocyte undergoes a slow but steady breakdown. Since DPN is the coenzyme for triosephosphate dehydrogenase in glycolysis, it was surmised that a failure in the mechanism responsible for the synthesis of DPN might be an initiating factor in the progressive impairment of the energy metabolism of the red blood cells during storage. Secondly, Stern (22) pointed out that of all the so-called "nuclear" enzymes, DPN pyrophosphorylase is the only one that is confined strictly to the nucleus. Rubinstein *et al.* (21) have shown that in the chicken erythrocyte, fumarase and malic dehydrogenase, though confined largely to the nucleus, are present also to an

¹ Manuscript received October 7, 1955.

Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec. This work was supported by the Defence Research Board of Canada, Grant number 9350-01, Project D 50-93050-01.

²Medical Research Fellow, National Research Council, Canada.

The following abbreviations are used throughout this paper: DPN, diphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; ATP, adenosine triphosphate; PP, inorganic pyrophosphate; NR, nicotinamide riboside; ADH-ase, alcohol dehydrogenase.

appreciable extent in the cytoplasm. Since fumarase and malic dehydrogenase are found also in the soluble fraction of the mature mammalian (nonnucleated) erythrocyte, the authors were unable to say whether in the evolution of the cell these enzymes were derived from the nucleus or from the cytoplasm of a nucleated precursor of the cell.

The precise nature of the "maturation" of the mammalian red blood cell has been a subject of controversy for many years. There are mainly three views as to the genesis of the erythrocyte. Some investigators believe that in the course of the maturation of the cell the nucleus is eliminated by "extrusion" (9); others hold the view that the normal mature red blood cell in the circulation represents a "cytoplasmic bud" nipped from a larger primitive red blood cell in the marrow (3); still others consider that the nucleus, in some way, disappears from within the immature erythrocyte by a process of karyolysis with or without karyorrhexis (5, 6).

To obtain further information on the fate of the nucleus during the maturation of the mammalian erythrocyte, a study was undertaken on the DPN pyrophosphorylase of the mature chicken erythrocyte, which retains the nucleus; the rabbit reticulocyte; and the mature erythrocyte from the rabbit and the human. Since DPN pyrophosphorylase activity is confined strictly to the nucleus of some somatic cells (22), its presence or absence in the chicken or mammalian erythrocyte might afford some information concerning the maturation of the mammalian red blood cell. If the enzyme activity be present in the nucleated erythrocyte and be retained in the nonnucleated erythrocyte, one may infer that in the development of the cell, nuclear fragments are dispersed but retained in the cytoplasm of the mature nonnucleated cell. On the other hand, if DPN pyrophosphorylase activity is not present in the mammalian erythrocyte, one may surmise that the nucleus is extruded from the cell during maturation. Although there is a class difference between the bird and the mammal, nevertheless the avian red blood cell is comparable with other somatic cells in many respects (20), and as other studies (17) in our laboratory have shown, is comparable with the mammalian reticulocyte. Furthermore, immature human erythrocytes are difficult to obtain free from other bone marrow elements, and, in any event, would not be available in sufficient quantities for our purpose.

Material and Methods

ATP and DPN were obtained from the Nutritional Biochemical Corporation and alcohol dehydrogenase was obtained from Sigma Biochemical Co. Lyophilized snake venom from the species *Crotalus adamanteus* was obtained from Ross Allen's Reptile Institute, Silver Springs, Florida, and was used as a source of 5'-nucleotidase for the preparation of nicotinamide riboside (NR).

NMN was prepared from DPN by means of a crude extract of potato nucleotide pyrophosphatase, prepared according to the method of Kornberg et al. (12). Since this preparation also contained a 5'-nucleotidase, a mixture of NMN and NR was obtained as cleavage products of DPN. NMN was

isolated from this mixture in a purified state by a method previously described (15). NR was prepared from the mixture of NMN and NR according to a method of Rowen *et al.* (20) by means of the 5'-nucleotidase from the snake venom.

Preparation of Erythrocytes

Chicken blood was collected with heparin as the anticoagulant. The samples were centrifuged and the plasma and "buffy layer" removed. The erythrocytes were washed three times with 0.9% sodium chloride, then diluted with this medium to the volume of the original blood sample. The red blood cells were then hemolyzed by alternately freezing the specimen in a dry-ice – alcohol mixture and thawing it in a bath at 37° C., the treatment being repeated three times. The hemolyzate was centrifuged at 3000 r.p.m. and the particulate fraction, containing nuclei and cellular membrane, was separated from the supernatant or stroma-free hemolyzate (SFH). The particulate fraction was washed with saline five or six times until almost white in appearance. To ensure the complete removal of finely divided particulate material the SFH was centrifuged further five or six times at 3000 r.p.m. Both the particulate fraction and SFH were then made up to the volume of the original hemolyzate with saline.

Reticulocytes were obtained from rabbits with an intense (95%) reticulocytosis produced by the injection of acetylphenylhydrazine. These specimens were kindly provided by a colleague, Mr. P. Ottolenghi. The reticulocytes were washed, hemolyzed, and fractionated into the particulate and SFH fractions as discussed above.

Rabbit and human erythrocytes were collected in heparin and similarly fractionated into the particulate fraction and the SFH.

Results

Chicken blood was studied first as a source of DPN pyrophosphorylase. With NMN and ATP as substrates, both the whole hemolyzate and the particulate fraction were found to synthesize DPN very readily (Table 1). Sodium fluoride appeared to inhibit the reaction in the whole hemolyzates, but not in the particulate fraction. The explanation for this apparent anomaly is that inorganic pyrophosphatase, the enzyme that catalyzes the hydrolysis of inorganic pyrophosphate, is confined entirely to the soluble portion (SFH) of the chicken, rabbit, and human red blood cells. This enzyme is inhibited in the presence of small concentrations of sodium fluoride (16). synthesis of DPN in the hemolyzates, therefore, inorganic pyrophosphate undergoes hydrolysis by the action of inorganic pyrophosphatase and thus is removed. But in the presence of sodium fluoride, the inorganic pyrophosphatase is inhibited, with the result that inorganic pyrophosphate accumulates and the synthesis of DPN (reaction [1]) is retarded. That sodium fluoride does not inhibit the enzyme directly is indicated by the observation that with the particulate preparation from chicken erythrocytes, DPN synthesis was not inhibited in the presence of sodium fluoride (Table I).

This interesting interrelationship between nuclear and cytoplasmic activity was tested in another way (Table II). A portion of the SFH or the cytoplasmic fraction, which by itself showed no synthetic activity, when combined with some of the particulate fraction considerably enhanced the activity of the latter. In the presence of sodium fluoride, however, the activity of the combined fractions was reduced to that obtained with the particulate fraction alone.

In order to demonstrate the reversibility of the reaction, that is the pyrophosphorolysis of DPN, PP and DPN were used as substrates (Table III) and the amount of DPN broken down was estimated at the end of the reaction period. In addition, to obtain initial values of DPN, the latter was incubated at 37° C. without the addition of PP but in the presence of the enzyme, to determine any breakdown of DPN by a DPN nucleosidase which may have been present. In the presence of sodium fluoride, the hemolyzate showed a much greater activity than in its absence. This enhancement again could be due only to inhibition of the inorganic pyrophosphatase, in which

TABLE I
DPN SYNTHESIS IN THE CHICKEN ERYTHROCYTE

Preparation	NaF	DPN synthesis (µM.)	Inhibition %
Hemolyzate	-	0.162	_
Hemolyzate	+	0.126	22
Particulate fraction	_	0.090	-
Particulate fraction	+	0.088	2

Incubation medium consisted of: 0.1 M ATP, 0.1 ml.; 0.002 M NMN, 0.2 ml.; 0.15 M MgCl₂, 0.1 ml.; 0.25 M glycyl-glycine buffer pH 7.4, 0.2 ml.; 2.0 M nicotinamide, 0.1 ml.; 0.01 M NaF, 0.1 ml.; H₂O to 1.0 ml. Enough enzyme was added to give approximately 40% removal of the NMN. Incubation 45 min. at 37° C. 1 ml. 10% trichloracetic acid was added to the incubation mixture. Following centrifugation, the filtrate was neutralized and DPN was determined by the alcohol dehydrogenase method (19). DPN synthesis expressed as the total amount in the 1.0 ml. of reaction mixture.

TABLE II

DPN synthesis by fractions prepared from chicken erythrocytes

Preparation	NaF	DPN (µM.)	Activation (%)
Particulate	-	0.059	_
SFH	-	0	_
Particulate + SFH	-	0.101	67
Particulate + SFH	+	0.055	- 6

Same conditions as for Table I, except that 0.1 ml. NMN was used as substrate. Incubation: two hours at 37° C.

circumstance inorganic pyrophosphate tends to accumulate and thus drive the equilibrium to the left (reaction [1]). Sodium fluoride had no effect on the pyrophosphorolytic activity of the particulate fraction. Nor did the SFH show any activity. When the SFH was combined with the particulate fraction, it caused a diminution in the activity of the latter, but in the presence of sodium fluoride, the activity was restored to the usual value. Thus again, as in the forward reaction, an interrelationship between the activity of the nuclear and cytoplasmic fractions was demonstrated.

With reference to the maturation of the erythrocyte, it was important to determine whether any pyrophosphorolytic activity could be elicited in the cytoplasmic fraction of the chicken erythrocyte. Erythrocyte fractions, therefore, were prepared, but this time the SFH was not made up to the original volume of the hemolyzate. The period of the experiment was extended, and the degree to which pyrophosphorolysis of DPN had occurred was expressed as the per cent of DPN that had undergone breakdown (Table IV). The undiluted SFH showed some activity, but after centrifugation at 25,000 × g for two hours, the activity was found to be diminished by 28%. activity was recovered in the gelatinous precipitate which had been thrown down during the centrifugation. One may conclude, therefore, that any activity of the SFH (cytoplasmic fraction) of the chicken erythrocyte probably may be attributable to the presence of finely divided nuclear material. Although the pyrophosphorylase is water-soluble, Hogeboom et al. (7) found that in the preparation of mouse liver nuclei, the enzyme cannot diffuse through the nuclear membrane. It would appear that in our nuclear preparations from chicken erythrocytes, some of the nuclei may have been ruptured and dispersed in colloidal form, thus imparting to the SFH the apparent capacity to synthesize DPN.

TABLE III

Pyrophosphorolysis of DPN in the chicken erythrocyte

Preparation	NaF	DPN (µM.)	Δ DPN $(\mu M.)$	DPN split
Hemolyzate	_	0.126	- 0.018	12.5
Hemolyzate	+	0.0198	-0.124	86.0
Particulate fraction	-	0.025	-0.155	86.0
Particulate fraction	+	0.022	-0.158	88.0
SFH	_	0.150	0	0
Particulate + SFH	-	0.108	-0.072	40
Particulate + SFH	+	0.011	- 0.169	94

Incubation medium consisted of: 0.004 M DPN, 0.1 ml.; 0.01 M PP, 0.1 ml.; 0.15 M MgCl2, 0.1 ml.; 0.25 M glycyl-glycine buffer, pH 7.4, 0.2 ml.; 2.0 M nicotinamide, 0.1 ml.; 0.01 M NaF, 0.1 ml. Sufficient enzyme was added to achieve almost total pyrophosphorolysis of DPN. Incubation: 45 min. DPN was determined as in Table I, and expressed as total amount in the reaction mixture.

A comparison was made of the activity of the whole hemolyzate from chicken erythrocytes with that of the cytoplasmic (SFH) and the particulate (nuclear) fractions, each of these fractions having been made up to the volume of the original whole hemolyzate. The SFH showed no activity, while that of the particulate fraction was 80% of the activity of the original whole hemolyzate (Table IV).

Throughout our study on the synthesis of DPN from NMN in the chicken erythrocyte, the maximum yield of DPN obtainable under a variety of conditions was equivalent to only 80% of the added NMN. Kaplan *et al.* (10) recently have demonstrated the presence of an α -isomer of DPN in crude and in purified preparations of DPN. This isomer is not hydrolyzable by the DPN nucleosidase of *Neurospora crassa* nor can it be reduced by alcohol dehydrogenase in the presence of ethanol. But like the β -isomer of DPN it reacts with cyanide. These authors found that purified preparations of DPN usually contain from 10 to 15% of the α -isomer. They postulate that the isomer has an α -ribosidic linkage whereas DPN has a β -linkage.

To ascertain whether our preparation of NMN contained the α -isomer along with the normal or β -form, the following experiments were carried out:

NMN was incubated, with and also without the addition of ATP, in the presence of an active particulate preparation of chicken erythrocytes (Table V). Nicotinamide was not added; hence any DPN that might be synthesized could be hydrolyzed by the DPN nucleosidase. Similarly, any hydrolysis of NMN itself would be detected in the control sample which consisted of NMN along with the particulate preparation of chicken erythrocytes, but without ATP. The quantity of cyanide-reacting material (NMN and DPN) was

TABLE IV

DISTRIBUTION OF DPN PYROPHOSPHORYLASE IN VARIOUS FRACTIONS
PREPARED FROM CHICKEN ERYTHROCYTE

Preparation	Pyrophosphorolysis (%)		Activity ounted for (%)	Fall in activity (%)
Hemolyzate	75			
Particulate	60		80	
SFH	0		0	
		Total	80	
SFH (undiluted)	29		-	_
SFH (undiluted, centrifuged)	21		72	28
Residue	5.9		20	_
		Total	92	

Conditions as given in Table III. In the first part, the duration of incubation was 30 min. The fractions were prepared as described under methods. In the second part, the SFH was not diluted. An aliquot was centrifuged at $25,000 \times g$. The supernatant and residue were assayed for DPN pyrophosphorylase; incubation time: two hours.

found to be the same whether ATP was present or not. In the presence of ATP, the amount of DPN, as measured by the alcohol dehydrogenase method, was found to be only 80% of the total amount of cyanide-reacting material. One interpretation of this situation is that equilibrium was reached when 80% of the theoretical maximum synthesis of DPN had taken place, the remaining material being unaltered NMN. There are two alternative explanations. The remaining cyanide-reacting material could represent an α -isomer of NMN, isolated along with the β -isomer in the preparation of this substance from DPN. This material would react with cyanide, but would not serve as a coenzyme for alcohol dehydrogenase. On the other hand, DPN pyrophosphorylase may be nonspecific in the sense that it is capable of catalyzing the synthesis of both the α - and the β -isomers of DPN from ATP and the α - or β -isomer of NMN, respectively. It is reasonable to suppose, therefore, that in this experiment the remaining cyanide-reacting material could be the α -isomer of DPN since in this case, as with the α - or β -isomers of NMN, the material would react with cyanide but could not serve as a coenzyme for alcohol dehydrogenase. Moreover, neither the α -isomer of NMN nor that of DPN would be hydrolyzed by DPN nucleosidase.

In order to eliminate the possibility that equilibrium was reached when only 80% of the theoretical maximum synthesis of DPN had taken place, a stroma preparation from rabbit erythrocytes was added to the reaction mixture. The stroma contains a very active DPN nucleosidase (1) hence any β -isomer of DPN synthesized would be removed by hydrolysis as fast as it was formed. In other words, the equilibrium of the reaction would be shifted as far to the right as possible (reaction [1]). Under these conditions (Table VI), with NMN and ATP present, no DPN could be detected with the alcohol dehydrogenase method, but there remained a small quantity of cyanide-reacting material equivalent to 21% of the amount of NMN originally added.

Thus, whereas under the conditions of the former experiment the equilibrium may have been reached when 80% of the theoretical amount of DPN had been synthesized, under the conditions of the latter experiment, the DPN

TABLE V Detection of the α -isomer of NMN

		CN-reacting		ADH-ase-reacting
NMN	ATP	material (µM.)	ADH-ase-reacting (µM.)	CN-reacting (%)
+	+	0.243	0.194	80
+	_	0.243	-	

Incubation medium consisted of: 0.003 M NMN, 0.1 ml.; 0.1 M ATP, 0.1 ml.; 0.25 M glycyl-glycine, pH 7.4, 0.2 ml.; 0.15 M MgCl₂, 0.1 ml.; H₂O to 0.6 ml. Exough enzyme added to drive the reaction to completion. Incubation: three hours. 0.6 ml. 5% trichloracetic acid added and mixture placed in boiling H₂O bath for 30 sec. Centrifuged. Supernatant neutralized with 2 N NaOH and aliquots taken for cyanide-reacting material (4) and for DPN assay (19).

TABLE VI DETECTION OF THE α -isomer of NMN

NMN	ATP	CN-reacting (µM.)	ADH-ase-reacting (µM.)	Fall in CN-reacting (%)
+	-	0.252	0	-
+	+	0.051	0	79

Same conditions as for Table V, but with the addition of 0.05 ml. of a rabbit erythrocyte stroma preparation containing an active DPN nucleosidase. Total incubation volume 0.65 ml. 0.55 ml. 5% trichloroacetic acid added after incubation.

would have been destroyed as rapidly as it was formed. Thus, all the NMN would have been used up. Theoretically, therefore, one should expect to find no remaining cyanide-reacting material in the latter case. The fact of the matter was, however, that there remained cyanide-reacting material equivalent to about 20% of the NMN originally present. Since in the latter experiment the material could not have been unaltered β -NMN, it is logical to conclude that it was either an α -isomer of NMN present as a contaminant in the starting material, or the α -isomer of DPN formed from α -NMN. A mixture of the two isomers of DPN may have been produced in the reaction. In either case, the NMN prepared from DPN by means of the potato nucleotide pyrophosphatase (12) appears to contain about 20% α -NMN. This value is comparable with 10-15% of α -DPN found in purified preparations of DPN by Kaplan *et al.* (10).

Rowen and Kornberg (20) demonstrated the synthesis of NMN from NR and ATP using crude fractions from hog liver. The synthesis, however, was found to proceed very slowly. Using the particulate fraction from chicken erythrocytes, or this fraction supplemented with SFH, we were unable to demonstrate the synthesis of DPN from NR and ATP.

Hemolyzates, prepared from the blood of rabbits with an induced reticulocytosis (50–90% reticulocytes), were tested as a source of DPN pyrophosphorylase. We were unable to demonstrate the pyrophosphorolysis of DPN with these preparations. There appeared, however, to be a consistent but slow and scanty synthesis of DPN in the presence of added NMN and ATP. The amount of DPN synthesized never was equivalent to more than 3-5% of the NMN added. These small yields are considered to be of doubtful significance.

With human erythrocytes, repeated attempts were made under a variety of conditions to demonstrate the synthesis or the pyrophosphorolysis of DPN. The efforts were uniformly unsuccessful. We are obliged, therefore, to conclude that under the conditions employed, DPN pyrophosphorylase activity is not present in the human erythrocyte.

Discussion

DPN pyrophosphorylase activity has been demonstrated in the chicken erythrocyte, the enzyme being confined to the particulate fraction. Any activity in the SFH was found to be attributable to the presence of finely divided particulate material which could be precipitated by high-speed centrifugation.

The particulate fraction of chicken erythrocytes consists of residues of nuclei and cell membrane. Rubinstein *et al.* (21) demonstrated that this fraction has succinoxidase activity and an active tricarboxylic acid system. With material from the erythrocytes prepared according to the method of Hogeboom *et al.* (8) the succinoxidase activity was found only in the readily sedimentable fraction. Chicken erythrocytes apparently do not contain mitochondria. Whereas the succinoxidase and the tricarboxylic acid systems are confined to the mitochondria in most somatic cells, these activities in the chicken erythrocyte appear to be confined to the nucleus.

From these considerations it may be concluded that the DPN pyrophosphorylase in the chicken erythrocyte may be associated with either the nucleus or the cell membrane. These two cell constituents in tissue homogenates, as Hogeboom et al. (7) have pointed out, are not readily separable. These authors suggest, furthermore, that the DPN pyrophosphorylase activity of their preparations of mouse liver nuclei probably resided in the nuclear rather than in the membrane material, since the membrane residues represent a relatively small fraction of the total sedimentable material. The same explanation applies to our nuclear preparations from chicken erythrocytes.

We were unable to demonstrate either the synthesis or pyrophosphorolysis of DPN in the human erythrocyte. The same thing can be said for the rabbit reticulocyte and erythrocyte, although with some of the preparations containing both types of cell, a slight amount of DPN appeared to have been formed. The synthetic activity, if any, was negligible.

The observation that the nuclear material from chicken erythrocytes contains an active DPN pyrophosphorylase raises the question whether, in the maturation of the mammalian erythrocyte, the nucleus actually undergoes fragmentation or dissolution (5, 6). If fragmentation takes place, one might expect to find some residual enzymatic activity in the mature, nonnucleated cell. Water-soluble components such as DPN pyrophosphorylase in particular (7) would tend to be dispersed in the cytoplasm. It would seem, then, that in the maturation of the mammalian red blood cell, the nucleus is either extruded from the cell in some mysterious fashion (9), or alternatively, the mature erythrocyte represents a cytoplasmic bud arising from a primitive precursor (2). Notwithstanding the class difference between the bird and the mammal, it is reasonable to suppose that there is a similarity at least in the enzyme components of the mature red blood cell of the bird and the nucleated precursors of mammalian erythrocytes.

Whether the gradual decrease in the concentration of DPN in the erythrocyte during storage as observed by Pappius et al. (18) contributes significantly to the progressive failure of the glycolytic system of the cell during storage is still an open question. The present study has indicated that the mature human erythrocyte cannot replenish DPN at least by synthesis from NMN and ATP.

Another mechanism for the synthesis of DPN has been demonstrated by Leder and Handler (13) who showed that by incubating intact human erythrocytes with nicotinamide over a long period there was an increase in the cellular content of pyridine nucleotides. Between 75 and 95% of this material appeared to be NMN, and the remainder, DPN. A synthesis of DPN similarly was noted with hemolyzates from human erythrocytes, supplemented with nicotinamide, ATP, glucose, and fructose diphosphate (14). In this case, the amount of DPN synthesized accounted for less than 10% of the total increment in the content of pyridine nucleotide. Whether the DPN was formed directly from nicotinamide or through a synthesis of NMN, is not known. The former process appears to be the more likely, since in our preparations from human erythrocytes there was no evidence of the formation of DPN from NMN and ATP, nor was there any evidence of the pyrophosphorolysis of DPN. In other words, the human erythrocyte apparently contains no active DPN pyrophosphorylase.

Hogeboom et al. (7) pointed out that the DPN synthesized in liver nuclei must traverse the nuclear membrane in order to participate in the glycolytic activity of the cytoplasm. Inorganic pyrophosphate, the other product of DPN synthesis, must also traverse the nuclear membrane. We have found that in the chicken erythrocyte, the inorganic pyrophosphatase is confined entirely to the soluble or cytoplasmic fraction of the cell. If inorganic pyrophosphate were to accumulate in the nucleus, and be unable to participate in other chemical reactions within the nucleus, its high concentration would tend to oppose the synthesis of DPN. But by being able to pass through the nuclear membrane, its destruction by hydrolysis in the cytoplasm is ensured. Furthermore, since DPN pyrophosphorylase appears to be confined to the nucleus, then it is unlikely that DPN will be broken down by pyrophosphorolysis, at least, in the cytoplasm. Thus, the nucleated erythrocyte offers an interesting example of the metabolic interplay between the nucleus and the cytoplasm, involving enzymes which apparently are confined to their respective cellular compartments and substrates which can traverse the membranes separating these compartments. The over-all picture is one of facilitated cellular function.

References

ALIVISATOS, S. G. A., KASHKET, S., and DENSTEDT, O. F. Can. J. Biochem. and Physiol. 34: 46. 1956.

^{2.} BALTUS, E. Biochim. et Biophys. Acta, 15: 263. 1954.

^{3.} Boström, L. Acta Med. Scand. 131: 303. 1948.

^{4.} COLOWICK, S. P., KAPLAN, N. O., and CIOTTI, M. M. J. Biol. Chem. 191: 447. 1951.

- 5. COOKE, W. E. Brit. Med. J. 1:433. 1930.
- 6. DAVIDSON, L. S. P. Edinburgh Med. J. 37: 425. 1930.
- 7. HOGEBOOM, G. H. and Schneider, W. C. J. Biol. Chem. 197:611. 1952.
- 8. Hogeboom, G. H., Schneider, W. C., and Striebich, M. J. J. Biol. Chem. 196:111.
- 9. HOWELL, W. H. J. Morphol. 4:57. 1890.
- 10. KAPLAN, N. O., CIOTTI, M. M., STOLZENBACH, F. E., and BACHEO, M. R. J. Am. Chem. Soc. 77:815. 1955.
- 11. KORNBERG, A. J. Biol. Chem. 182:779. 1950.
- 12. KORNBERG, A. and PRICER, W. E., JR. J. Biol. Chem. 182:763. 1950.
- LEDER, I. G. and HANDLER, P. J. Biol. Chem. 189: 889. 1951.
 LEDER, I. G. and HANDLER, P. Phosphorus metabolism. Vol. 1. Edited by McElroy, W. D. and Glass, B. The Johns Hopkins Press, Baltimore, Md. 1951. p. 421.
- 15. MALKIN, A. and DENSTEDT, O. F. Can. J. Biochem. Physiol. 34:141. 1956.
- 16. NAGANNA, B. and MENON, V. K. N. J. Biol. Chem. 174: 501. 1948.
- 17. OTTOLENGHI, P., RUBINSTEIN, D., and DENSTEDT, O. F. Unpublished.
- 18. Pappius, H. M., Andreae, S. R., Woodford, V. R., and Denstedt, O. F. Can. J. Biochem. Physiol. 32:271. 1954.
- 19. RACKER, E. J. Biol. Chem. 184: 313. 1950.
- 20. Rowen, J. W. and Kornberg, A. J. Biol. Chem. 193: 497. 1951.
- 21. RUBINSTEIN, D. and DENSTEDT, O. F. J. Biol. Chem. 204:623. 1953.
- 22. STERN, H. Science, 121:144. 1955.

THE METABOLISM OF THE ERYTHROCYTE

XII. DIPHOSPHOPYRIDINE NUCLEOTIDE NUCLEOSIDASE OF THE RABBIT ERYTHROCYTE1

By A. MALKIN² AND O. F. DENSTEDT

Abstract

Evidence is presented in support of the hypothesis that in the hydrolysis of DPN by the DPN nucleosidase of rabbit erythrocyte stroma, the substrate is attached to the enzyme at more than one site. Cleavage of DPN at the nicotinamide-ribose bond is inhibited by nicotinamide, ADP, and adenine. Considering the nature of the inhibition and the extent of the inhibition, it is suggested that DPN attaches to the enzyme surface at the quaternary nitrogen of the nicotinamide and at the pyrophosphate group of the DPN Additional support for this supposition accrues from the observation that NMN, a cleavage product of DPN, can serve as a substrate for DPN-ase but the rate of hydrolysis is much slower than that with DPN.

Introduction

In recent studies on the DPN-ase³ of erythrocytes, Alivisatos et al. (1) showed that a large group of purine and pyrimidine compounds are effective inhibitors of this enzyme. In view of these findings it seemed possible that a further insight into the nature of the DPN-DPN-ase complex might be afforded by studying the effect of various components of the DPN molecule on DPN-ase. Rabbit erythrocyte stroma was used as a source of the enzyme.

Material and Methods

DPN, adenine, adenosine, AMP, ADP, and ATP were obtained from the Nutritional Biochemical Corporation and alcohol dehydrogenase, from Sigma Biochemical Co. Hydrolyzed snake venom from the species Crotalus adamanteus was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, and used as the source of 5'-nucleotidase. NMN was prepared from DPN according to the method of Kornberg et al. (5), by means of a crude preparation of potato nucleotide pyrophosphatase. Since this enzyme preparation contained a 5'-nucleotidase, mixtures of NMN and NR were obtained as cleavage products. NMN was removed in a purified state by the following procedure.

The mixture of NMN and NR was passed through a Dowex 50 \times 12 cation exchange column, 200-400 mesh, and 10 cm. X 2 cm., previously washed with 2 M acetic acid and water. The final water washings showed no absorption at 256 m µ (8).

¹Manuscript received September 30, 1955.

Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec, This work was supported by the Defence Research Board of Canada, Grant number 9350-01, Project D 50-93050-01.

^{*}Medical Research Fellow, National Research Council, Canada.

*The following abbreviations are used throughout this paper: DPN-ase, diphosphopyridine nucleotide nucleosidase; DPN, diphosphopyridine nucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; APP, adenosine diphosphate; ATP, adenosine triphosphate; AMP, adenosine-5'-phosphate; PP, inorganic pyrophosphate.

NMN was then eluted with 0.01 M acetate buffer pH 4.9 at a flow rate of 2 ml./min. The presence of NMN was followed by the observation of absorption values at 256 m μ and, after the addition of 1.0 M sodium cyanide to small aliquots of the eluate, at 325 m μ (2). The absorption curve showed symmetrical peaks. The combined eluates were acidified to pH 2.0 with concentrated hydrochloric acid and the volume reduced by vacuum distillation at < 30° C. The pH was finally adjusted to 6.0 and the volume brought to 12 ml. The concentration of the NR moiety of the NMN, thus prepared, was estimated by the cyanide method. The total phosphate, obtained after hydrolysis of the compound with 70% perchloric acid, and inorganic phosphate, were measured according to the method of Fiske and SubbaRow (3). The NR: phosphate ratio was found to be 1:1.03. The final preparation of NMN was 0.003 molar.

NR was prepared from a mixture of NMN and NR by the method of Rowen and Kornberg (10) using the preparation of snake venom referred to above. The final preparation of NR was found to contain no phosphate after hydrolysis with 70% perchloric acid. The material was 0.001 M as determined by the cyanide method, using an extinction coefficient of 6.3×10^6 cm.² \times mole⁻¹ at 325 m μ .

In studies on the cleavage of NMN and NR by rabbit erythrocyte stroma, the cyanide method was used throughout. The hydrolysis of DPN and the inhibition of this reaction were studied using the alcohol dehydrogenase Trichloracetic acid (5%) was added to the reaction method of Racker (9). mixture following the incubation. After centrifugation, the filtrate was neutralized with 2 N NaOH with phenol red as indicator. Trichloracetic acid, similarly neutralized, served as a blank. To an aliquot of filtrate were added 0.3 ml. 95% ethanol, 1.6 ml. 0.1 M sodium pyrophosphate, and H₂O Readings were made at 340 m μ . After the addition of 82.5 γ of to 2.9 ml. alcohol dehydrogenase to each cuvette, the contents were mixed by inversion, and readings were again taken. The reduction of DPN was usually complete within five minutes. This method gave readily reproducible results. An extinction coefficient of 6.3 × 106 cm.2 × mole-1 at 340 m \u03c0 was used in the calculation of the results.

Preparation of Rabbit Erythrocyte Stroma

Rabbit venous blood was collected in heparin. The sample was centrifuged and the plasma and the 'buffy layer' were removed. The red cells were washed three or four times with 0.9% sodium chloride and brought to the original volume of the blood specimen with this medium. The cells were then hemolyzed by alternate freezing (in alcohol – dry ice) and thawing (at 37° C.), three times repeated. The stroma was separated from the stroma-free hemolyzate by centrifugation at 3000 r.p.m. The stroma residue was washed three or four times with isotonic sodium chloride and the volume was adjusted to that of the original hemolyzate with saline.

Results

NMN was tested first as a substrate for the DPN-ase of the rabbit erythrocyte stroma (Table I). NMN was hydrolyzed at the nicotinamide-ribose bond but the rate of cleavage was very slow compared with that with DPN. The rate of hydrolysis of DPN under comparable conditions is about 200 times as rapid. Leder and Handler (6) observed that NMN, synthesized by the human erythrocyte, was hydrolyzed about as rapidly as exogenous DPN by either the rat brain DPN-ase or the human erythrocyte DPN-ase.

Both nicotinamide and adenine inhibit the cleavage of NMN by DPN-ase (Table I).

Handler and Klein (4) had noted that although DPN was rapidly hydrolyzed following incubation with a broken cell preparation of rat brain, NR under similar conditions remained intact. We found that NR was not hydrolyzed by an active stroma preparation from rabbit erythrocytes.

Several compounds including ATP and the components of DPN, namely, NR, adenine, adenosine, AMP, ADP, and PP were tested for their effect on DPN-ase (Table II). As Alivisatos *et al.* observed (1) adenine was found to be an effective inhibitor of the enzyme while no inhibition occurred with

TABLE I
Inhibition of rabbit erythrocyte stroma DPN-ase, using NMN as substrate

Velocity (µmoles)	Compd. used as inhibitor	Inhibition (%)
0.045	_	_
_	Nicotinamide	100
0.015	Adenine	67

Incubation medium consisted of: NMN, 0.001 M; 0.03 M acetate buffer pH 4.75; adenine 6.7 \times 10⁻³ M, nicolinamide 3.3 \times 10⁻¹ M. Total volume of reaction mixture 0.6 ml. Incubation: three hours. 0.4 ml. 1 M acetate buffer, pH 4.25, added and mixture heated in boiling water bath for one minute.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON RABBIT ERYTHROCYTE DPN-ASE,
USING DPN AS SUBSTRATE

Compound used as inhibitor	Molarity of inhibitor	Inhibition (%)
NR	1.67 × 10 ⁻⁴	0
Adenine	5 × 10 ⁻³	40
Adenosine	3 × 10 ⁻²	0
AMP	1.87×10^{-3}	0
ADP	1.87×10^{-3}	20
ATP	1.87×10^{-2}	0
PP	2.5 × 10 ⁻²	0

Incubation medium consisted of: DPN, 6.7×10^{-4} M; 0.03 M acetate buffer, pH 4.25. Total volume 0.6 ml. Incubation: 10 min. at 37° C. 0.4 ml. 5% trichloroacetic acid added and mixture heated in boiling water bath 30 sec. Sufficient enzyme was added to give approximately 50% hydrolysis of DPN in 10 min.

sodium pyrophosphate. However, whereas these authors demonstrated a slight inhibition with adenosine and AMP, we could show no inhibition with these compounds. The discrepancy may have arisen from a difference in the conditions under which the reaction was carried out, or, alternatively, from a difference in the assay method. We would suggest that the alcohol dehydrogenase method used in this study is more specific than the cyanide method used by the previous authors. The inhibition of DPN-ase activity by ADP, but not by ATP, was a rather unexpected finding. In the light of these and other observations it would appear that a DPN-DPN-ase complex is formed according to the mechanism described below.

Discussion

In a study previously carried out in our laboratory, Alivisatos et al. (1) had shown that the hydrolysis of DPN by DPN-ase is inhibited by nicotinamide, adenine, and a number of purine derivatives, notably the dimethyl purines theobromine and theophylline. The evident affinity of the enzyme for free nicotinamide and adenine suggested that DPN is capable of assuming a two-point attachment to the enzyme surface through the terminal components nicotinamide and adenine. Furthermore, this dual attachment appears to be essential for the splitting off of the nicotinamide by the enzyme.

The present authors have made the following additional observations which

complicate the picture:

1. DPN-ase is capable of splitting nicotinamide from nicotinamide mononucleotide (NMN) which does not contain adenine. Furthermore, the hydrolysis of NMN can be inhibited by nicotinamide and also by adenine.

2. Neither adenosine nor nicotinamide riboside (NR) inhibits DPN-ase.

3. DPN-ase is inhibited by ADP but not by AMP or ATP.

From the first of these observations it would appear that the presence of a terminal adenine component in the molecule is not essential to the action of DPN-ase in splitting the nicotinamide-ribose linkage. The finding that the hydrolysis of this bond in NMN can be inhibited with adenine as well as with nicotinamide suggests that these compounds may compete for the same site on the enzyme surface rather than for different sites as postulated by Alivisatos et al. (1).

The powerful inhibition of DPN-ase by adenine and other substituted purines, and the absence of inhibition by adenosine, AMP, or ATP suggest that in order for inhibition to occur the purine and particularly the nitrogen in position 9 in the molecule must be free. The only exception to this apparently is the inhibition by ADP. It is improbable that the adenine component of the latter compound plays a part in the inhibition. It is noteworthy that the quaternary nitrogen of nicotinamide must be free since NR does not inhibit the enzyme.

An alternative explanation for the inhibition by ADP is that the pyrophosphate component may be the active group. If so, this group must possess distinctive properties compared with those of free pyrophosphoric acid, the

monophosphate group of AMP or the triphosphate in ATP, all of which compounds cause no inhibition. To test the validity of the hypothesis that organic pyrophosphate as in ADP may constitute a point of attachment to the enzyme, a compound such as nicotinamide-ribose-pyrophosphate would be useful since this substance should be hydrolyzable by DPN-ase at about the same rate as DPN. Furthermore, it should be a still more effective inhibitor of DPN-ase than free nicotinamide, when DPN is used as the substrate.

The DPN-DPN-ase system affords an unusually favorable one for the study of the nature of the enzyme-substrate complex. The flavoproteins, though not strictly comparable, may lend themselves to the same type of inhibitor study for elucidating the nature of the attachment between the flavine adenine dinucleotide and the protein component. Michaelis (7), in his study of Warburg's yellow enzyme, pointed out the probability of a multiple attachment between the flavin prosthetic group and the apoenzyme. He favored the idea that fixation of the flavin moiety occurs at the alloxan and the azine rings. But as Michaelis himself stated (7): "It is remarkable, however, that without the phosphate group the flavin dye is not bound at all to the apoenzyme". In this sense, an analogy might be drawn between the DPN-DPN-ase system and the attachment of the flavin prosthetic group to the apoenzyme in the flavoproteins.

It is evident that the picture of enzyme-inhibitor attachment is still confused, and it may be that the spatial concept of simple physical attachment between chemical groupings on the inhibitor and sites on the enzyme surface will not adequately explain the phenomena observed.

References

- 1. ALIVISATOS, S. G. A., KASHKET, S., and DENSTEDT, O. F. Can. J. Biochem. Physiol. 34:46. 1956.
- 2. COLOWICK, S. P., KAPLAN, N. O., and CIOTTI, M. M. J. Biol. Chem. 191: 447. 1951.
- Fiske, C. H. and Subbarow, Y. J. Biol. Chem. 66: 375. 1925.
 Handler, P. and Klein, J. R. J. Biol. Chem. 144: 453. 1942.
- 5. KORNBERG, A. and PRICER, W. E., JR. J. Biol. Chem. 182:763. 1950.
- 6. LEDER, I. G. and HANDLER, P. J. Biol. Chem. 189: 889. 1951.
- MICHAELIS, L. The enzymes. Vol. II. Pt. 1. Edited by Sumner, J. B. and Myrbäck, K. Academic Press, Inc., New York. 1951. p. 50.
- 8. PLAUT, G. W. E. and PLAUT, K. A. Arch. Biochem. and Biophys. 48: 189. 1954.
- 9. RACKER, E. J. Biol. Chem. 184: 313. 1950.
- 10. ROWEN, J. W. and KORNBERG, A. J. Biol. Chem. 193: 497. 1951.

STUDIES ON HUMAN ADRENAL STEROIDS

THE EFFECT OF CORTICOTROPIN ON COMPONENTS OF THE FREE AND CONJUGATED PLASMA C21 ADRENAL STEROID FRACTIONS¹

BY C. M. SOUTHCOTT, S. K. GANDOSSI, A. D. BARKER, H. E. BANDY, HAMISH McIntosh, and Marvin Darrach

Abstract

The free and conjugated adrenal steroid fractions of peripheral plasma from 12 normal human males were studied. Specimens were withdrawn before and after the administration of corticotropin. Hydrocortisone was identified chemically and some evidence was obtained for the presence of corticosterone. Hydrocortisone levels showed a marked but variable increase after corticotropin treatment. In some cases the administration of corticotropin resulted in the appearance of an unconjugated compound which may have been a tetrahydro derivative of cortisone or hydrocortisone. A method for studying the conjugated fraction was developed and preliminary data indicated that four components were present in some specimens after hydrolysis with \$\textit{P}_{\textit{cl}}glucuronidase. The level of these conjugates appeared to increase after treatment with corticotropin. In general, the response to a given dose of corticotropin showed considerable individual variation in the plasma levels of the components of both the free and conjugated corticosteroid fractions.

Introduction

Existing evidence seems to indicate that hydrocortisone is a constituent of human peripheral blood (14, 6) and that both hydrocortisone and corticosterone may be present in human adrenal venous blood (16). Experimental data also suggest that human plasma contains other free C_{21} steroids (2, 16) as well as conjugated C_{21} steroids which can be hydrolyzed with acid (9) and β -glucuronidase (3). Although limited knowledge exists concerning the precise identity and amounts of the adrenal steroids and their metabolites in human blood, it has been clearly demonstrated that human urine contains an array of both free and conjugated steroids of adrenal origin (7, 13, 12, 15). A small portion of these may be unchanged native hormones (21, 5); the remainder are probably metabolites of the original steroids, resulting from tissue activity (2, 15). Therefore, it would be expected that blood should contain a mixture of the original adrenocortical hormones and some of their metabolites.

Several investigators have contributed to an evolution of good methods for determining the C_{21} adrenal steroids in biological materials (4, 20, 1, 19, 10). The problem of analyzing human plasma, however, remains complicated because of the limited volume of fresh blood available from a human subject, the low levels of adrenal steroids and their metabolites likely to be present, and the micro character of the methods which, because of numerous sources of error, casts some doubt on the most carefully derived quantitative data.

¹Manuscript received October 11, 1955. Contribution from the Department of Biochemistry, Faculty of Medicine, University of British Columbia, and Clinical Investigation Unit, Shaughnessy Hospital, Vancouver, Canada. This report outlines part of an investigation into the effect of corticotropin on constituents of the free and conjugated plasma C_{21} adrenal steroid fractions. These were separated from a single specimen, the conjugates digested with β -glucuronidase and the components of each fraction isolated on paper chromatograms. Quantitative methods were employed wherever possible. The study was conducted on a series of plasma specimens from 12 normal human males taken before and after a course of intravenous corticotropin for 24 hr.

Experimental

Collection of Blood and Administration of Corticotropin

Peripheral blood was withdrawn at 8.00 a.m. from fasting normal male donors, 20–30 yr. of age. About 175 ml. were collected in 70 ml. of dextrose – sodium citrate solution² and separate samples were taken for cell volume determinations. This procedure was repeated the following morning after 20 International Units of corticotropin³ in 1 liter of 5% glucose⁴ had been given to each individual by intravenous drip for 24 hr. The citrated blood samples were refrigerated for about six hours and then centrifuged in the cold at 2000 r.p.m. for 40 min.; the plasma and cells were separated and stored in the freezer.

Separation of Conjugated and Unconjugated Plasma C21 Adrenal Steroid Fractions

The citrated plasma samples were dialyzed and extracted in 70 ml. portions by methods similar to those described by Zaffaroni (19) and Axelrod and Zaffaroni (1). Each portion was mixed with 70 ml. of water⁵ and 70 ml. of methanol and then transferred to a dialyzing bag prepared from washed Visking casing.⁶ A glass rod, padded at both ends with washed casing strips, was inserted lengthwise into the dialyzing bag. The bag was then tied and placed in a separatory tube (1, 19) containing 150 ml. of chloroform and the tube was rotated mechanically at 12 r.p.m. (19) for 47 hr. at room temperature.

The chloroform phase, containing the dialyzed unconjugated adrenal steroid fraction (1), was separated from the 40% aqueous methanol which contained conjugated compounds subsequently hydrolyzed by β -glucuronidase.

Purification and Chromatography of the Unconjugated C21 Adrenal Steroid Fraction

Neutral Fraction

The 40% methanol was washed with 4 × 25 ml. portions of chloroform and these were added to the main extract. Earlier observations had revealed that the chloroform, at this stage, frequently contained unknown ultraviolet

*Acthar-Armour Laboratories, Chicago, Ill.

²Dex-trate-Formula No. 1, Baxter Laboratories of Canada, Ltd., Acton, Ont.

⁴Travenol—Five per cent dextrose w/v in water, Baxter Laboratories of Canada, Ltd., Acton, Ont. ⁵Water and the organic solvents were distilled in all glass equipment.

^{*}Diameter, 28 mm. Washed in three changes of water and six of 40% methanol, changes being made twice daily.

(U.V.) absorbing substances of plasma origin which moved downward in the toluene - propylene glycol system at the same rates as cortisone and hydrocortisone. Since these interfering compounds were found to be soluble in alkali, as illustrated in Fig. 4, they were extracted from the chloroform with 3 × 15 ml. portions of 0.1 N sodium hydroxide. The combined alkali washings were back-extracted with a 10 ml. portion of chloroform which was then added to the main extract. After the chloroform solution was washed with 1×10 ml. of 0.01 N hydrochloric acid and 3×10 ml. of water, it was dried over sodium sulphate, passed through a sintered glass filter, and evaporated to dryness in vacuo at 40° C. The residue was transferred quantitatively with chloroform and methanol through a sintered glass filter into a small tube and dried at 40° C, under a stream of nitrogen. Chromatographic separation of the residue was carried out on paper7 in the toluene - propylene glycol system (4, 20) for 45 hr. beside 10 gamma standards of cortisone and hydrocortisone. After the overflow from the first chromatogram was evaporated to dryness in vacuo at 40° C., the residue was rechromatographed for three hours in the toluene - propylene glycol system or for six hours in the benzeneformamide system (4, 20) beside a 10 gamma standard of corticosterone.

Acidic (and Phenolic) Fraction

The sodium hydroxide washings from the above procedure were immediately brought to pH 7 with dilute hydrochloric acid and extracted with 5×15 ml. portions of chloroform. These were combined, washed with 3×5 ml. of water, dried, and evaporated. This residue was chromatographed in the same way as described above for the neutral fraction.

Digestion, Purification, and Chromatography of the Conjugated C21 Adrenal Steroid Fraction

Using phenolphthalein monoglucuronide as substrate, it was established that neither spleen nor bacterial β -glucuronidase was active in 40% methanol but showed full activity in 10% methanol. It was also shown that bacterial β -glucuronidase preparations gave 80% greater hydrolysis of phenolphthalein monoglucuronide at 47° C. than at 37° C. Therefore the plasma conjugated corticosteroid fractions were digested by the following procedure.

The 40% aqueous methanol phase was concentrated *in vacuo* at 40° C. from about 150 ml. to 20–30 ml. and buffered with acetate to give a concentration of 0.15 M and a pH of 6.2. Bacterial β -glucuronidase⁸ was added to provide 62.5 units/ml., the solution was incubated at 47° C. for 17 hr. and then extracted with 5 \times 20 ml. portions of chloroform. These extracts were combined, processed, and chromatographed as before.

Analysis of the Paper Chromatograms

The toluene – propylene glycol chromatograms were dried for one hour and the benzene-formamide chromatograms for two hours at 80° C. Contact

⁷All chromatograms in this study were prepared on Whatman No. 1 filter paper which had been washed by a descending flow of methanol for three days.

Sigma Chemical Company, St. Louis, Missouri.

prints of each chromatogram were made on Kodak Velox F-5 paper, with U.V. light which had passed through a Corning No. 9863 (8) glass filter. The photographs revealed the position of the U.V. absorbing zones and were used as patterns for cutting measured areas of the unknowns, standards, and paper blanks from the chromatograms. These were cut into small pieces and eluted with 1 ml. of ethanol in a small stoppered tube on a mechanical shaker for 20 min. at room temperature. After the solutions were transferred to 1 ml. cuvettes, absorption curves were prepared over the 230-300 m u range. 10 When the unknown showed a peak in the 238-242 m µ region, it was assumed that a Δ^4 -3-keto C_{21} steroid was present. The quantity was then estimated by comparison of the optical density at the peak of the curve with that of the appropriate chromatographed standard after the subtraction from each of the blank reading for paper of equal area.

Immediately after the U.V. analysis, the quantitative blue tetrazolium (B.T.) reaction of Mader and Buck (11), for detecting primary α -ketol groups, was applied in the following manner. Aliquots (0.3 ml.) of the unknown, standard, and paper blank were withdrawn from the cuvettes and each was mixed with 0.35 ml. of 0.24% tetramethyl ammonium hydroxide in ethanol and 0.35 ml. of 0.12% blue tetrazolium in ethanol. A reagent blank was prepared at the same time. The mixtures were then agitated on a mechanical shaker for 30 min. at room temperature. After 30 min., optical densities of the solutions were measured at 520 m µ against the reagent blank. A second quantitative value was then calculated as before.

The Porter-Silber method (17) served as a third quantitative analysis for determining the 17,21-dihydroxy-20-ketosteroids. Aliquots of the unknown, standard, and paper blank were removed from the microcuvettes, transferred quantitatively through sintered glass, and divided into two equal parts. Each of these was taken to dryness under nitrogen at 40° C. To one part was added 1 ml. of the blank reagent11 and to the other 1 ml. of the blank reagent containing phenylhydrazine hydrochloride12. The solutions were shaken mechanically for 15 min. and allowed to stand overnight at room temperature. Optical density readings were then recorded for each solution against the corresponding reagent blank over the 300-500 m µ range and after appropriate corrections were made, absorption curves were prepared for the unknown and standard. Providing these were typical Porter-Silber absorption curves, the quantity of the unknown was calculated by comparison of its optical density at 410 m with that of the standard.

Table I shows typical quantitative data given by each of the three methods when applied to eluates of F 1.0 zones derived from human plasma. Unlike the U.V. and B.T. procedures, the Porter-Silber method has consistently given very low readings for paper blanks, as illustrated in Fig. 1; it is therefore,

⁹Supplied by Pyrocell Manufacturing Company, New York, N.Y.

19 The cuvettes were centered in the normal light beam of the Beckman D.U. Spectrophotometer.

11 Blank reagent—38 ml. water, 62 ml. concentrated sulphuric acid, and 50 ml. ethanol.

12 Phenythydrazine hydrochloride (43 mgm.) dissolved in 100 ml. blank reagent.

TABLE I

APPLICATION OF THREE QUANTITATIVE METHODS TO THE F 1.0 (HYDROCORTISONE) ZONES FROM HUMAN PLASMA

(Gamma per 100 ml. plasma-as found)

Donor*	U.V. method†	B.T. method‡	Porter-Silber method
A	26.9	27.0	21.2
В	24.8	23.4	22.4
C	27.9	26.3	27.1
D	20.7	20.3	15.9

Specimens withdrawn immediately after 24 hr, stimulation with 20 I.U. corticotropin. Reaction based on Δ^4 -3-keto ring A group.

Reaction based on a-ketol side chain group.

considered a more reliable assay for 17,21-dihydroxy-20-ketosteroids. Nevertheless, the application of all three methods to a single eluate gives valuable information concerning the identity and amount of the steroid under study.

The remainder of the ethanol eluates of the unknown and standard zones, or pooled eluates of several unknown zones believed to contain identical substances, were filtered, taken to dryness, and acetylated at room temperature with acetic anhydride and pyridine. The dried residues were chromatographed, photographed, and eluted as before. These eluates were examined by the quantitative U.V. method and, when sufficient material was available, aliquots were taken for further analysis by the quantitative B.T. and Porter-Silber methods. Finally, the remaining solutions of the unknown and

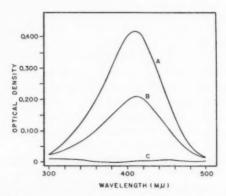


Fig. 1. Porter-Silber absorption curves. A. Chromatographed acetate derivative of pooled F 1.0 (hydrocortisone) zones from human plasma. B. Chromatographed acetate derivative of hydrocortisone standard. C. Paper blank. The low paper blank readings given by the Porter-Silber method afford a more accurate quantitative procedure for determining low levels of 17,21-dihydroxy-20-ketosteroids than either the U.V. or B.T. methods, both of which give higher and more variable paper blank readings.

Reaction based on 17,21-dihydroxy-20-keto side chain group.

standard, containing at least 10 gamma each, were taken to dryness and the residues were dissolved in 1 ml. of sulphuric acid (18). After two hours at room temperature, absorption curves were prepared over the 230–600 m μ range.

All remaining parts of the chromatograms were immersed in blue tetrazolium solution (4)¹³. When reducing zones appeared, the paper was allowed to dry at room temperature in the dark, and the positions and intensities of the zones were recorded photographically on Kodabromide F-4 by making contact prints in green light.

Recovery and Control Experiments

Periodically, the equipment, materials, and reagents were checked by blank runs. Providing the washing and reagent purification procedures were followed as described, the chromatograms were free of U.V. absorbing and B.T. reducing zones.

Several qualitative recovery experiments were conducted using pooled human plasma and various combinations of 10 gamma quantities of corticosteroids ranging in polarity from hydrocortisone to desoxycorticosterone. No evidence was obtained from these experiments to suggest that the process was responsible for chemical alterations to the added steroids.

Four recovery control experiments with 20 gamma quantities of hydrocortisone and corticosterone added to 50 ml. portions of plasma gave average recovery yields of 54.6% for hydrocortisone and 52.3% for corticosterone. The variation in each case was approximately \pm 5%. Similar experiments reported by Zaffaroni (1, 19) gave much higher recovery yields for hydrocortisone, but in these studies 1000 gamma of steroid was added per 15 ml. of blood. Zaffaroni (19) also showed that the time recovery curve for extraction of steroids by dialysis levelled off at about 36–48 hr. Similarly, at the lower steroid levels in the experiments reported here, an increase in the time of dialysis from two to five days had no significant effect on the yield of hydrocortisone. For example, in one experiment a recovery of 50.8% was obtained after two days' dialysis and 56.8% after five.

It was also demonstrated, by quantitative recovery experiments, that when the chloroform extracts were washed quickly with sodium hydroxide, acid, and water, no loss of hydrocortisone occurred. In duplicate experiments 20 gamma of hydrocortisone was added to 300 ml. portions of chloroform which were divided into equal parts. One part was washed and dried by the usual method and then both were evaporated, the residue being chromatographed and analyzed by the quantitative U.V. method. Identical yields were obtained from washed and unwashed samples. Furthermore, chloroform extracts of the neutralized washings after evaporation and chromatography showed no U.V. absorbing nor B.T. reducing zone opposite the hydrocortisone standard.

 ^{13}Two parts aqueous 0.2% blue tetrazolium: one part aqueous 10% sodium hydroxide. Prepared immediately before use.

Results

THE UNCONJUGATED PLASMA C21 ADRENAL STEROID FRACTIONS

Neutral Fraction

A typical set of U.V. photographs of the chromatograms, prepared from the plasma unconjugated C₂₁ adrenal steroid fraction of one of the 12 individuals studied, is illustrated in Fig. 2. Here, also, are designated the zones and regions which were examined in detail as described below. The position of the unknown zones is referred to the nearest standard, this being either cortisone (E), hydrocortisone (F), or corticosterone (B); the number following is the ratio of the distance traveled by the unknown as compared with the standard.

i. The 45 Hr. Toluene - Propylene Glycol Chromatograms

Starting zone.—Photographs of each of the 45 hr. toluene – propylene glycol chromatograms showed U.V. absorbing zones at the starting line. Since blank runs gave no absorption in this area, the starting zones apparently contained substances of plasma origin and were, therefore, removed for further study. No consistent change in the appearance of the starting zones from the same individual accompanied the administration of corticotropin.

Start to F 1.0 region.—All photographs of this region showed either well-defined U.V. absorbing zones or hazy areas. No consistent change in the density of the zones followed the administration of corticotropin. The start to F 1.0 regions were removed from the chromatograms and immersed in a blue tetrazolium solution. A B.T. positive zone occurred only on 5 of the 12 regions from specimens taken after treatment with corticotropin. Since tetrahydrohydrocortisone and tetrahydrocortisone standards also appeared within this region, these five B.T. positive zones might have been one or both of these steroids. There was no relation between the position or intensity of the B.T. positive and U.V. absorbing zones in this region.

F 1.0 zone (hydrocortisone).—A U.V. absorbing zone appeared beside the hydrocortisone standard on each chromatogram and a marked increase in the size and density of the zone occurred in each case after the 12 subjects received corticotropin. Each zone, derived from plasma samples taken after treatment with corticotropin, was eluted and analyzed by the quantitative U.V. and B.T. methods. In all cases the solutions gave U.V. absorption curves with peaks at 241.5 m μ and positive reactions with blue tetrazolium. The quantitative data are given in Table II. The F 1.0 zones from six of the samples taken before treatment were similarly analyzed. The remainder were examined by the U.V. method only and then pooled to obtain sufficient material for identification of the zones. A comparison of the effect of corticotropin on the circulating hydrocortisone levels of these six individuals, determined by both the U.V. and B.T. methods, is shown in Fig. 5. The remainder of the eluates from specimens taken before corticotropin treatment were pooled separately from those taken afterwards.

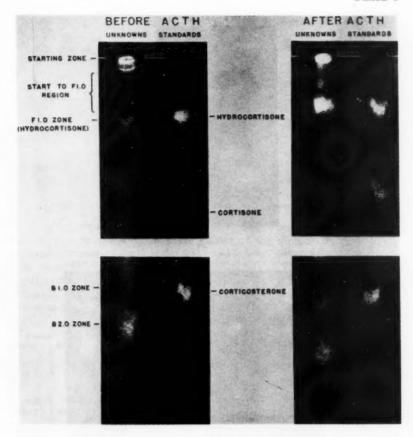


Fig. 2. U.V. contact photographs of neutral unconjugated plasma C_{21} adrenal steroid fractions. Specimens were taken from the same individual before and after the administration of corticotropin.

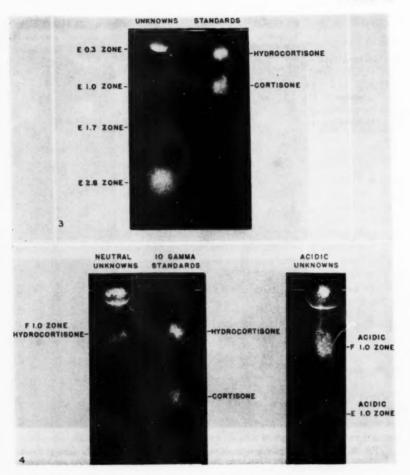


Fig. 3. Green light contact photograph of a blue tetrazolium treated chromatogram showing four zones from a pool of three human plasma specimens. These compounds were isolated from the conjugated fraction after hydrolysis with β -glucuronidase.

FIG. 4. U.V. contact photograph of the unconjugated plasma C21 adrenal steroid fraction, showing the acidic (or phenolic) U.V. absorbing zones removed by sodium hydroxide washings.

TABLE II

Hydrocortisone plasma levels of normal human males after the intravenous administration of 20 International Units of corticotropin for 24 hr.

(Gamma per 100 ml. plasma-as found)

Donor	U.V. method	B.T. method
1	32.4	25.8
2	26.8	24.2
3	20.4	14.4
4	23.2	20.5
5	14.7	10.3
6	25.0	16.7
7	18.2	16.5
8	21.4	19.6
9	22.8	21.8
10	16.2	13.4
11	17.3	15.4
12	17.7	19.8

residue, after chromatography, appeared as single zones beside a hydrocortisone acetate standard. Ethanol eluates of the three zones gave U.V. absorption curves with peaks at 241.5 m μ . Aliquots of these also gave typical Porter–Silber absorption curves. The quantitative data from the two methods showed equivalent values when the unknowns were calculated as hydrocortisone acetate. A quantitative B.T. analysis was also made on an aliquot of the eluted acetate from the specimens collected after corticotropin treatment

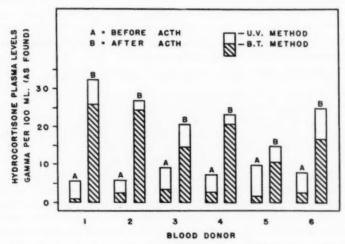


Fig. 5. Effect of corticotropin on hydrocortisone plasma levels of six normal human males.

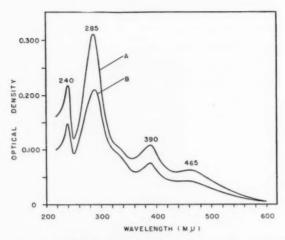


Fig. 6. Absorption curves of sulphuric acid chromogens. A. Chromatographed acetate derivative of pooled F 1.0 (hydrocortisone) zones from human plasma. B. Chromatographed acetate derivative of hydrocortisone standard.

and the value given, when calculated as hydrocortisone acetate, corresponded with the U.V. and Porter–Silber data. Finally, the residue from this sample and the hydrocortisone acetate standard showed identical absorption curves in sulphuric acid, as illustrated in Fig. 6. Thus, the F 1.0 zones were shown to contain hydrocortisone.

E 1.0 region.—None of the 24 photographs of this area revealed a U.V. absorbing zone.

Neither U.V. absorbing nor B.T. positive zones occurred elsewhere on the 45 hr. toluene – propylene glycol chromatograms.

ii. The Three Hour Toluene - Propylene Glycol Chromatograms

B~1.0~zone.—A U.V. absorbing zone which travelled at about the same rate as the corticosterone standard appeared on 23 of the 24 photographs. Ethanol eluates of each B 1.0 zone gave high optical density readings at 230 m μ and the absorption curves fell off smoothly through the 240 m μ region. In two cases peaks occurred at 273 m μ . These were probably due to contamination from the B 2.0 zone which did not always separate entirely from the B 1.0 zone. The administration of corticotropin had no obvious effect on the B 1.0 zones as judged from the appearance of their densities on the U.V. photographs or from their plasma concentrations based on optical density readings at 230 m μ . Eluates of the B 1.0 zones gave negative B.T. readings. The dried residue from filtered eluates of the remainder of four B 1.0 zones were acetylated and chromatographed on paper for three hours in the benzene-formamide system. No spot occurred opposite the cortiocosterone acetate

standard. The residue had descended at the same rate as before acetylation, with a small amount separating into the B 2.0 position. Pooled aliquots of 19 eluates of the B 1.0 zones, when rechromatographed in benzene–formamide for six hours beside a corticosterone standard, separated into four U.V. absorbing zones. One of these appeared opposite corticosterone and gave a positive B.T. reaction; the one above and two below the level of the corticosterone standard gave negative B.T. reactions. It would appear from these observations that a small amount of corticosterone could have been present in one or more of the B 1.0 zones. However, insufficient material was available for either its positive identification or for quantitative determinations. Therefore, the effect of corticotropin on this material could not be determined.

B~2.0~zone.—Of the 24 chromatograms, 21 contained an U.V. absorbing zone at approximately the B 2.0 position. Ethanol eluates of these gave absorption curves with peaks at 273 m μ . A comparison of the plasma concentrations of this material, based on the optical density readings at 273 m μ , showed no consistent change after the administration of corticotropin. Eight of the eluates selected at random and tested separately did not reduce blue tetrazolium. The remainder of four eluates were pooled and the chromatographic behavior of the residue remained unchanged after treatment with acetic anhydride and pyridine. No additional U.V. absorbing nor B.T. reducing zone appeared on the chromatogram. A similar pool of 17 eluates, when rechromatographed for six hours in the benzene–formamide system, descended as a single zone.

Acidic (and Phenolic) Fraction

The 45 Hr. Toluene - Propylene Glycol Chromatograms

Fig. 4 shows a typical U.V. photograph of this fraction with zones at the starting line, and approximately at the F 1.0 and E 1.0 positions.

Acidic starting zone.—The chromatograms contained variable amounts of U.V. absorbing material and the administration of corticotropin had no apparent effect on the density of the zones. They were not examined in further detail.

Acidic F 1.0 zone.—Of the 24 U.V. photographs, 21 showed zones in this area. The ethanol eluates of three of these, selected at random, gave absorption curves with high optical densities at 230 m μ , valleys at 245 m μ , and peaks at 272 m μ . The occurrence and density of the zones had no apparent relation to the administration of corticotropin.

Acidic E 1.0 zone.—Of the 24 U.V. photographs, seven showed zones at approximately this position. Eluates gave smooth absorption curves descending from a high optical density at 220 m μ , with no deflection through the 240 m μ region. Pooled zones did not separate on a 144 hr. chromatogram developed in the toluene – propylene glycol system. Also, the chromatographic behavior did not change after mild acetylation. There was no correlation between corticotropin treatment and the appearance of these zones.

THE CONJUGATED PLASMA C21 ADRENAL STEROID FRACTIONS

A pooled sample of the 40% methanol solutions, containing dialyzed chloroform insoluble materials from the plasma of three individuals who had received corticotropin, was concentrated in vacuo, buffered with acetate, and divided into three equal parts. One of these was refrigerated, another incubated at 47° C., and the third digested with β -glucuronidase. A blank containing buffer and enzyme served as a reagent control. At the end of 17 hr., the four solutions were extracted with chloroform and the residues chromatographed as before. The chromatograms showed no U.V. absorbing zones. Each was then immersed in blue tetrazolium solution. The 45 hr. toluene – propylene glycol chromatogram of the sample digested with β -glucuronidase developed two large and two small zones. These are illustrated in Fig. 3. The largest occurred at positions E 0.3 and E 2.8 and the smallest at E 1.0 and E 1.7. None of the other chromatograms showed a B.T. reducing zone.

A pool of the 40% methanol fractions from the plasma of the same three individuals before treatment with corticotropin was analyzed for conjugated compounds. As before, the paper chromatograms contained no U.V. absorbing zones. The 48 hr. toluene – propylene glycol chromatogram, when treated with blue tetrazolium, gave only two small positive zones at E 0.3 and E 2.8.

Thus, it was established that the four compounds reacting with blue tetrazolium had been hydrolyzed by the action of β -glucuronidase and the amounts of each increased after the subjects received corticotropin.

Additional observations were made on the 40% methanol fractions of plasma samples withdrawn from a different individual, before and after adrenal stimulation with corticotropin. Again, none of the chromatograms showed U.V. absorbing zones. The sample taken before corticotropin treatment showed no B.T. positive zones but the sample taken after revealed two large and two small zones as before. The largest appeared at E 0.13 and E 2.4 and the smallest at E 1.0 and E 1.6. A third set of 40% methanol fractions from another individual were analyzed similarly, but in this case corticotropin treatment only produced an increase in density of the B.T. positive zone at E. 0.2. No other B.T. positive zones appeared on the chromatograms.

In a further experiment, the plasma conjugated fractions from three individuals who had received corticotropin were pooled and digested with β -glucuronidase as before. An aliquot containing one-third of the chloroform soluble residue was chromatographed beside the remaining two thirds. The chromatogram showed no U.V. positive zones. A strip containing the smaller portion of the residue was cut from the chromatogram and immersed in blue tetrazolium. Again, two large and two very small zones appeared at the usual positions. The E 0.2 region from the path of the larger portion was then removed, eluted with ethanol, and tested by the quantitative Porter–Silber method. The reaction mixture gave a typical absorption curve for a 17,21-dihydroxy-20-ketosteroid.

Further studies on the chemical nature of these zones are in progress.

Discussion

The scope of this study included both free C21 adrenal steroids and those conjugated with β -glucuronic acid. It was limited, however, to compounds separating on the chromatograms within the range bounded by tetrahydrohydrocortisone and 11-desoxycorticosterone. Furthermore, only steroids having a Δ^4 -3-keto ring A group, a primary α -ketol side chain, or both, could be detected by the methods employed.

Of the free C21 adrenal steroids which appeared to be present in human plasma, hydrocortisone was positively identified; some evidence was obtained for the presence of corticosterone and possibly tetrahydrohydrocortisone or tetrahydrocortisone. In addition, four compounds hydrolyzed by

 β -glucuronidase were isolated.

In general terms the experiments have demonstrated that the adrenal response to intravenous corticotropin in the normal human male results in an increase of circulating hydrocortisone, the appearance in some cases of unconjugated material which could be a tetrahydroderivative of cortisone or hydrocortisone, or both, and an increase in the components of the fraction conjugated with β -glucuronic acid. It would also appear that the concentration of any of these circulating steroids in response to a given dose of corticotropin may vary with the individual.

Acknowledgments

These investigations have been assisted by grants-in-aid for equipment and technical help from the Canadian Arthritis and Rheumatism Society, the National Cancer Institute of Canada, and the National Health Grants. The steroid standards were kindly donated by Dr. Augustus Gibson, Merck and Company Inc., Rahway, New Jersey.

References

AXELROD, L. R. and ZAFFARONI, A. Arch. Biochem. and Biophys. 50: 347. 1954.
 BAYLISS, R. I. S. and STEINBECK, A. W. Biochem. J. 54: 523. 1953.
 BONGIOVANNI, A. M. J. Clin. Endocrinol. and Metabolism. 14: 341. 1954.

Bongiovanni, A. M. J. Clin. Endocrinol. and Metabolism, 14: 341. 1954.
 Burton, R. B., Zaffaroni, A., and Keutmann, E. H. J. Biol. Chem. 188: 763. 1951.
 Burton, R. B., Zaffaroni, A., and Keutmann, E. H. J. Biol. Chem. 193: 769. 1951.
 Bush, I. E. and Sandberg, A. A. J. Biol. Chem. 205: 783. 1953.
 Dorfman, R. I. Recent Progr. Hormone Research, 9: 55. 1954.
 Haines, W. J. Recent Progr. Hormone Research, 7: 255. 1952.
 Klein, R., Papadatas, C., Fortunato, J., and Byers, C. J. Clin. Endocrinol. and Metabolism, 15: 215. 1955.
 Levy, H. and Kushinsky, S. Recent Progr. Hormone Research, 9: 357. 1954.
 Mader, W. J. and Buck, R. R. Anal. Chem. 24: 666. 1952.
 Marrian, G. F. Recent Progr. Hormone Research, 9: 303. 1954.
 Mason, H. L. Recent Progr. Hormone Research, 9: 267. 1954.
 Nelson, D. H., Samuels, L. T., Willardson, D. G., and Tyler, F. B. J. Clin. Endocrinol. 11: 1021. 1951.

NELSON, D. H., SAMUELS, L. T., WILLARDSON, D. G., and TYLER, F. B. J. Clin. Endocrinol. 11: 1021. 1951.
 ROBERTS, S. and SZEGO, C. M. Ann. Rev. Biochem. 24: 543. 1955.
 ROMANOFF, E. B., HUDSON, P., and PINCUS, G. J. Clin. Endocrinol. and Metabolism, 13: 1546. 1953.
 SILBER, R. H. and PORTER, C. C. J. Biol. Chem. 210: 923. 1954.
 ZAFFARONI, A. J. Am. Chem. Soc. 72: 3828. 1950.
 ZAFFARONI, A. Recent Progr. Hormone Research, 8: 51. 1953.
 ZAFFARONI, A. and BURTON, R. B. J. Biol. Chem. 193: 749. 1951.
 ZAFFARONI, A., BURTON, R. B., and KEUTMANN, E. H. Science, 111: 6. 1950.

"REFLEX" ANURIA IN THE DOG!

By Sydney M. Friedman, Roland W. Radcliffe, J. E. H. Turpin, and Constance L. Friedman

Abstract

The effects of various surgical manipulations on the function of the separate kidneys was studied in the dog. The application of a clamp to one renal artery produced vasoconstriction of varying severity in the contralateral kidney. The introduction of a venous catheter into the renal vein by passage upward from the femoral vein similarly caused renal vasoconstriction. The combination of manipulations involved in preparing the renal pedicle for later renal artery clamping with the passing of renal venous and ureteral catheters frequently produced oliguria or complete anuria. Since such nociceptive stimuli can cause renal vasoconstriction and, if sufficiently severe, antidiuresis, it is inferred that these mechanisms bear directly on the problem of traumatic anuria.

Despite an extensive literature, the mechanism of clinically observed "renal shutdown" remains obscure. The variety of names applied to the condition and the numerous causal factors which have been suggested imply that perhaps a final common pathway remains to be discovered.

In an illuminating analysis of the problem, Oliver and his colleagues (6) have suggested renal ischemia as one of the major pathogenetic mechanisms. They conclude that clamping the renal artery to produce ischemia is a valid experimental method for approaching the problem. This, of course, does not answer the question of how renal ischemia arises clinically where no clamp is present.

Recently, we reported on the sequence of events that follows release of a clamp in uninephrectomized dogs (3). In this study, we noted that a functional ischemia persisted for several hours beyond the time when circulation was restored to the renal artery. The present work was undertaken to pursue the cause of this persistent vasoconstriction in the hope of clarifying the spontaneous mechanisms causing renal ischemia in man. Our plan was to clamp the renal artery of one kidney, using the unclamped contralateral kidney as the control. Early in the study it became apparent that renal reflexes were involved and these became the prime object of the investigation. Evidence is presented for the existence of renal reflexes, probably "venorenal reflexes" capable of inducing vasoconstriction of varying degrees of severity up to and including complete renal shutdown.

Methods

Mongrel dogs exceeding 30 lb. in weight and of both sexes were used. The general procedures are considered here while special procedures will be indicated for each experiment.

¹Manuscript received October 7, 1955. Contribution from the Department of Anatomy, The University of British Columbia, Vancouver, British Columbia. This work was carried out under Defence Research Board Grant 9310-48, Project Number D50-93-10-48. Intravenous nembutal anesthesia (20–30 mgm./kgm.) was used for all experiments, with foreknowledge of the functional changes it may induce (4). A free airway was ensured by endotracheal intubation. Infusions of glucosesaline through the left saphenous vein were started immediately the animal was anesthetized.

The abdomen was opened mid-ventrally. Polyethylene catheters were inserted into the ureters two to three inches below the renal pelvis.

Where required, venous catheters were inserted in two stages. In the first stage, with the abdomen closed, one catheter was passed down from the jugular vein and one up from the femoral vein. Both were guided into the inferior vena cava to a point estimated to be opposite the renal veins. In the second stage, with the abdomen open, the upper catheter was guided into the left renal vein, the lower catheter threaded around the acute bend into the right renal vein.

The right femoral artery was cannulated with an 18 gauge needle and connected through a Sanborn electromanometer to a viso-cardiette. A side-arm connection permitted sampling of arterial blood as required. Blood pressure records were obtained throughout the procedure, but being non-contributory are not dealt with in this communication.

At the completion of the operation a priming dose of para-aminohippurate (PAH) and creatinine was given, followed by a maintenance infusion, as used by Phillips et al. (7). Renal function was studied using standard technics for creatinine and PAH as described by Selkurt (8). Extraction fractions were determined by sampling blood from the femoral artery and simultaneously from catheters in each renal vein. No facilities were available for refrigerated centrifuging so that the error of this omission (7) is present in our PAH data. All clearance data are referred to surface area using the formula of Cowgill and Drabkin (2).

Experimental

Experiment 1. Effects of a Right Renal Artery Clamp on Renal Functions. Catheters in Both Renal Veins

Seventeen dogs were used in this study. The procedure was as described. Detailed function studies were started as soon as all manipulations were completed, the last of these being the final insertion of catheters into the renal veins. The results obtained are presented in Tables I and II.

As shown in Table I, manipulation to this stage frequently induced marked oliguria or even complete anuria. Since this occurrence in eight dogs precluded any further study of renal function, these animals, after a minimum of six hours of observation, were classed as failures and discarded. In this we followed the usual practice and only later did we realize that the occurrence of a renal shutdown on manipulation was itself an important clue to the nature of the condition under study.

In the remaining nine animals a satisfactory urine output was obtained after completion of the procedure. It is worth noting that "successful" preparations were obtained only where local anesthesia was infiltrated around the

TABLE I

Urine output of left unclamped kidney before and after clamping right kidney

	Peri	od of clamping		Period of cla	imp removal
Dog No.	Average preclamp	Immediate postclamp	Average postclamp	Immediately prior to	Immediately following
H-6b	Negligible				
H-14	Negligible				
H-15	Irregular to negli	gible			
H-16	Anuria				
H-18	Anuria				
H-25	Anuria and death	1			
R-05	Anuria				
R-06	Anuria left kidne	У			
H-7	0.23	0.12	0.15	0.23	0.14
H-8	0.17	0.00	0.12	0.08	0.16
R-01	0.77	0.65	0.65	0.60	0.60
H-9	0.21	0.40	1.00	1.50	1.27
H-10	0.07	0.23	0.35	0.44	0.33
H-13	0.32	0.42	0.92	1.17	1.12
H-17	0.46	0.53	1.00	1.28	1.47
H-19	0.28	0.40	0.49	0.73	0.86
H-20	0.35	0.60	0.87	0.91	1.10
Average	0.32	0.37	0.62	0.77	0.78
S.E.	0.06	0.07	0.11	0.16	0.16

Eight of 17 were complete failures.

Three of nine show fall in urine output on clamping.

S.E. =
$$\sqrt{\frac{\sum x^2 - [\bar{x}(\sum x)]}{n(n-1)}}$$
. This calculation is omitted in all groups of less than five cases.

renal pedicle and especially around the femoral vein and inferior vena cava. (Initially 2% procaine was used but later abandoned because it interferes with PAH determinations and 0.2% xylocaine was substituted.)

Following one to one and one-half hours of basal observation, right kidney function was eliminated by clamping the renal artery for two hours, this vessel having been exposed and tagged beforehand. Upon release of the clamp, this kidney remained anuric or occasionally extremely oliguric for the remainder of the study period, which extended for an additional three to five hours. This agrees with our previously reported finding that following release of a renal artery clamp the kidney tends to remain non-functioning for several additional hours despite the restoration of free pulsatile flow in the artery. Accordingly, no function is recorded in the tables for the right kidney.

Renal function on the left was considerably upset by application of the contralateral clamp. In Table I it appears, on the average, that urine output remained the same as before. In three of the dogs under consideration, however, a sharp fall was observed, while in six there was an immediate increase in output. Similarly, upon release of the clamp, the output of the left kidney declined in two cases while in the others there was a trend towards an increase, although no function was returned to the right kidney by release of the clamp.

RIGHT KIDNEY. CATHETERS IN BOTH RENAL VEINS

TABLE II
FUNCTIONS OF LEFT UNCLAMPED KIDNEY BEFORE AND AFTER CLAMPING

	Pe	riod of clamp	ing	Period of cla	amp removal
	Average preclamp	Immediate postclamp	Average postclamp	Immediately prior to	Immediately following
Urine vol., cc./min.	0.21	0.40	1.00	1.50	1.27
	0.07	0.23	0.35	0.44	0.33
	0.46	0.53	1.00	1.28	1.47
	0.28	0.40	0.49	0.73	0.86
	0.35	0.60	0.87	0.91	1.10
Average	0.27	0.43	0.74*	0.97	1.00
S.E.	0.07	0.06	0.13	0.19	0.21
Cer, cc./min./m.3	23.5	32.1	32.1	32.1	35.3
	28.4	28.2	28.2	28.2	21.4
	33.5	28.8	29.4	30.0	29.5
	23.1	22.0	22.6	23.2	26.1
	33.9	31.6	32.8	34.8	35.3
Average	28.5	28.5	29.0	29.7	29.5
S.E.	2.2	1.9	1.8	1.2	2.7
CPAH, cc./min./m.2	117.8	85.7	85.7	85.7	88.6
	147.2	-	79.0	79.0	64.6
	142.0	104.0	103.5	103.0	85.0
	97.3	93.0	92.7	88.5	90.3
	136.7	115.0	112.0	109.8	116.7
Average	128.2	99.4*	94.6*	93.2	89.0
S.E	9.2	_	5.9	5.7	8.3
RPF PAH,	174.3	105.2	105.2	105.2	110.4
cc./min./m.3	204.0		99.0	-	
	176.0	162.0	154.5	147.0	120.0
	169.5	162.4	169.1	175.9	148.4
	225.3	190.6	187.4	184.1	141.1
Average	189.8	155.0	143.0*	153.0	130.0
S.E.	1.1	_	1.7	-	_
E., %	69.3	68.7	68.5	68.5	71.9
	3.0	5.2	5.5	6.0	3.6

^{* =} Significant change.

In five of the nine "successful" dogs full clearance studies were carried out including in four, arteriovenous extraction determinations. As shown in Table II, the act of clamping was followed by a significant increase in contralateral urine output. This was accomplished without change in the filtration rate but with a significant fall in PAH clearance (C_{PAH}). This latter fall represented a true decline in renal plasma flow which persisted even after removal of the clamp.

The results of this experiment indicate that clamping the renal artery on one side causes a "reflex" vasoconstriction on the opposite side. The compensatory increase in function which might have been expected on the unclamped side was not observed.

Experiment 2. Effects of a Right Artery Clamp on Renal Functions. No Venous Catheters

The previous experiment demonstrated that C_{PAH} provided an adequate indication of the renal plasma flow on the unclamped side. Further, it was our impression that the insertion of venous catheters was traumatic and responsible, at least in part, for the frequency of complete renal shutdown. Accordingly, this experiment was carried out in five dogs with the omission of venous catheterization and consequently without the determination of PAH extraction. The findings, again limited to the left kidney which alone was functional, are presented in Table III.

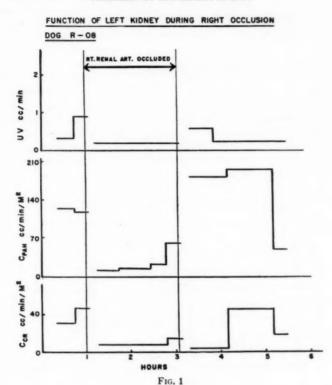
As before, clamping the right renal artery caused a significant reduction in renal plasma flow on the opposite side. The degree of reduction varied but in one case (Fig. 1) amounted to almost complete ischemia. The filtration rate tended to remain steady (Fig. 2), although in the above-cited severe response it too was reduced. Urine output declined slightly upon contralateral clamping.

TABLE III

FUNCTIONS OF LEFT UNCLAMPED KIDNEY BEFORE AND AFTER CLAMPING RIGHT KIDNEY. NO VENOUS CATHETERS

	Pe	riod of clamp	ing	Period of cla	imp removal
	Average preclamp	Immediate postclamp	Average postclamp	Immediately prior to	Immediately following
Urine vol., cc./min.	0.95	0.90	1.03	1.10	1.80
	0.45	0.10	0.10	0.17	0.62
	1.35	1.45	1.64	1.75	1.50
	0.50	0.45	0.40	_	0.30
	0.95	0.35	0.47	0.47	0.77
Average	0.84	0.65	0.73	0.87	1.00
S.E.	0.16	0.23	0.27	_	0.28
C _{cr.} cc./min./m. ²	55.1	45.4	58.3	47.4	65.3
	38.5	7.4	10.0	15.7	5.7
	52.5	53.0	50.7	50.3	53.7
	39.7	43.5	42.1	_	46.3
	50.1	53.5	62.8	47.7	53.1
Average	47.2	40.5	44.8	40.3	44.8
S.E.	3.3	8.5	9.3	_	10.2
CPAH, cc./min./m.3	107.6	90.5	135.4	110.0	121.4
- I make the control of the control	127.7	6.1	22.2	60.1	181.0
	160.3	128.7	114.4	107.7	136.1
	189.5	158.8	123.5		172.5
	150.9	116.1	120.5	129.3	176.2
Average	147.2	100.0*	103.2*	102.0	157.4*
S.E.	14.0	25.9	20.5	_	12.1

^{* =} Significant change.



In Table IV the values for Experiments 1 and 2 are compared. Venous catheterization, the major variable between the groups, clearly appears to have reduced urine output, filtration rate, and C_{PAH}. Furthermore, in the numerous experiments on some 40 dogs carried out since Experiment 1 was completed and without venous catheterization, no oliguric "unsuccessful" preparations have been encountered.

TABLE IV

Initial renal function with and without venous catheters

Preparation	Urine vol., cc./min.	C _{er} , cc./min./m. ³	C _{PAR} , cc./min./m. ²	No. of
Ureteral and venous catheters	0.27 ± 0.07	28.5 ± 2.2	128.2 ± 9.2	5
Ureteral catheters only	0.84 ± 0.16	47.2 ± 3.3	147.2 ± 14.0	5

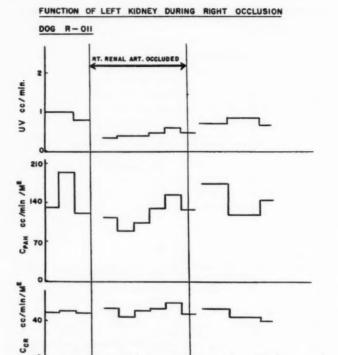


Fig. 2

HOURS

Experiment 3. Control Studies of Left Kidney Function

Since manipulation of the venous segment below the renal veins caused vasoconstrictive changes to the extent of anuria in some dogs and clamping one renal artery caused contralateral vasoconstriction, it was decided to study a series of animals without manipulation as controls and then a series subjected to controlled manipulation. Five dogs were used for the control study.

TABLE V
Functions of left kidney without manipulation of right kidney

	Urine vol., cc./min.	cc./min./m.2	C _{PAH} , cc./min./m. ²	No. of dogs
Average, 1st hr.	0.55 ± 0.27	38.4 ± 4.8	120.4 ± 26.4	5
Average, 2nd hr.	0.68 ± 0.23	37.4 ± 4.0	128.4 ± 33.5	5
Average, 3rd hr.	0.65 ± 0.22	40.9 ± 5.5	121.7 ± 29.1	5

Clearance determinations were carried out every 20 min. over a three hour period in three dogs and over a five hour period in two dogs. Table V presents the average data for the three hour study. Renal function remained unchanged in all dogs studied.

Experiment 4. Functions of Separate Kidneys Before and After Controlled Manipulations

Four dogs were studied in this experiment. Following ureteral cannulation, basal observations were carried out through three or four 20 min. clearance periods. The right renal artery was then dissected free but no clamp applied. Four clearance periods were recorded following this manipulation. Venous catheters were then passed into the inferior vena cava to a point opposite the renal veins, one from above through the jugular vein, one from below through

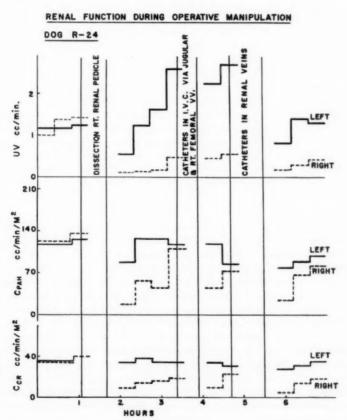


Fig. 3

 $\label{thm:constraints} TABLE\ VI$ Functions of separate kidneys before and after manipulations $\label{eq:constraints} Dogs\ R23\text{-}R26$

	Cleani	ng right renal	pedicle		of catheters nal veins
	Average premanip.	Immediate postmanip.	Average postmanip.	Premanip.	Postmanip
Urine vol., cc./min.					
Left kidney	0.57	0.29	0.30	_	_
	1.25	0.62	1.89	2.80	0.92
	0.24	0.10	0.10	0.62	0.27
	1.35	0.57	0.79	0.02	0.27
Average	0.85	0.39*	0.77		
Right kidney	0.72	0.45	0.46	_	
reight mane)	1.28	0.12	0.16	0.57	0.17
	0.26	0.10	0.10	_	-
	0.95	0.17	0.31	0.37	0.30
Average	0.80	0.21*	0.26*	1.09	0.41*
Cer, cc./min./m.2					
Left kidney	54.8	52.0	47.8		-
	46.5	33.6	24.1	29.4	28.4
	42.7	22.5	19.3		
	56.8	60.8	57.5	60.6	27.0
Average	50.2	42.2	37.2		
Right kidney	53.3	48.0	43.8		_
angue manay	37.7	8.7	15.0	23.0	4.8
	37.1	1.3	5.4	-	_
	55.9	18.4	24.6	45.2	28.8
Average	46.0	19.1*	22.2*	39.5	22.2*
C _{PAH} , cc./min./m. ²					
Left kidney	135.8	152.4	126.4	_	_
	121.8	93.6	99.0	85.8	78.4
	1772	74.3	40.3	442.2	
	141.6	90.7	112.9	117.7	66.5
Average	144.1	102.7*	94.6*		
Right kidney	113.0	127.4	114.6	_	
	128.5	16.7	58.5	74.0	25.2
	123.7	27.8	33.1		
	133.9	25.5	63.7	102.0	72.5
Average	124.8	49.3*	67.5*	94.9	60.6*

^{* =} Significant change.

the right femoral vein. Two more clearance periods were recorded at this time. The catheters were then threaded into the respective renal veins and three more clearance periods recorded. Data concerning the first and last manipulations are shown in Table VI. Placing the catheters into the inferior vena cava produced only slight changes; these are shown in Fig. 3 for dog R-24.

Surgical dissection of the right renal pedicle produced a sharp decline in urine output from both kidneys, more marked on the manipulated side. This change was accompanied by a decrease, on the average, in the filtration rate from both kidneys, marked on the manipulated side, moderate on the other. Although urine output decreased on the left side in each of the four individual dogs, this was not accompanied by a change in filtration rate in two of them. Similarly, in comparison of individual cases with the average data, it is noteworthy that on the manipulated side one dog showed no fall in filtration rate although urine output was halved. These findings suggest the partial independence of the effects of manipulation on urine flow and filtration rate.

A sharp fall in C_{PAH} occurred on the manipulated side with a lesser but still considerable fall on the other. In one dog C_{PAH} remained unchanged.

Similar effects were noted at the time of the last manipulation in the two dogs studied. The observed reductions were again more apparent on the right side. This is undoubtedly related to the fact that considerable manipulation is required to thread the lower catheter into the right renal vein.

Fig. 3 illustrates the case of one dog in which C_{PAH} for the right kidney dropped sharply when the lower catheter was threaded into the inferior vena cava. Data to be reported separately show that this is a reflection of the fact that below the union of the iliac veins the renal "reflexes" here described appear to be primarily linked with the homolateral kidney.

Discussion

From these experiments it has been shown that vasoconstrictive "reflexes" may be set up in the unclamped vessel as well as the clamped. These "reflexes" may also be elicited by the manipulations involved in preparing the experimental animal. As the nature of these "reflexes" and their "triggers" became clearer, controlled procedures were set up to demonstrate them.

It seems that a major trigger for these "reflexes" is located in the lower venous segment, i.e., the inferior vena cava below the renal veins, together with the iliac and femoral veins. For convenience, we propose to group the phenomena under the collective term "venorenal reflexes". This is not meant to exclude trigger areas other than those in veins from later consideration.

So far it appears that varying degrees of stimulation result in varying degrees of vasoconstriction. Since a decline in C_{PAH} occurred most regularly, with or without change in creatinine clearance (C_{er}) or in urine output, we postulate that the initial effect is primarily efferent arteriolar vasoconstriction accompanied, if the response is greater in degree, by a fall in urine output. A still greater response is marked by afferent constriction and a fall in C_{er} . The

reflex is partially crossed, since manipulation on one side exerts its effects mainly on the same side but also, to a lesser degree, on the opposite side.

The most severe response is characterized by anuria or marked oliguria. Although this type of response was obtained accidentally in Experiment 1, we have since determined that it may be deliberately produced.

Study of the continuous blood pressure records showed no relation between the renal events and the blood pressure level.

The clinical literature is replete with instances of anuria or oliguria following even relatively simple manipulations of the genitourinary tract and "lower venous segment". So persistently have these occurrences been noted that the term "reflex anuria" has acquired a certain significance for the physician. In discussing the phenomenon, Smith (9) has tended to dismiss it as probably non-specific, largely because of the lack of control and the paucity of experimental support. Yet recently, Handley and Moyer (5) stated: "Excessive handling of the kidney during exposure of the renal artery is likely to induce a variable degree of renal ischemia". The experiments reported here show that "reflex anuria" is a definite entity that can be produced under experimental conditions.

Many investigators have noted "reflex anuria" as a side issue and have discarded the preparations as unsuitable for the pursuit of the main line of their investigations. It seems, however, scarcely to be a side issue when, as in our first experiment, 8 of 17 dogs became anuric or oliguric and remained so for several hours.

Several authors have recently been concerned with the role of nerves in the formation of the urinary load. Block *et al.* (1) observed that electrical stimulation of the renal nerves frequently produced a short-lived anuria. Because of the transient nature of the response, they concluded that "the present study does not support the view that anuria or oliguria of any significant duration is so produced". We suggest that the transient response was due to the inadequacy of the stimulus rather than the unimportance of the mechanism. In fact these workers did succeed in producing reflex oliguria for as long as 70 min.

At present we are emphasizing the lower venous segment as a source of vasoconstrictive renal stimuli. This is based on the observations with manipulation of venous catheters. It is also based on direct evidence to be reported separately concerning "adequate" stimuli. It is not intended to exclude other trigger areas, possibly in arteries, from separate consideration, nor have we yet presented proof for the predominance of nervous over humoral factors, although an extension of the findings of Block *et al.* (1) would strongly suggest that nerves are involved at least to some extent.

For the present our evidence shows only that nociceptive stimuli cause renal vasoconstriction and, if sufficiently severe, marked antidiuresis. Since the antidiuresis may be of sufficient degree to be classed as oliguria or even anuria it seems fair to infer that these mechanisms may bear on the problem of traumatic anuria.

References

- 1. BLOCK, M. A., WAKIM, K. G., and MANN, F. C. Am. J. Physiol. 169: 670. 1952.
- 2. COWGILL, G. R. and DRABKIN, D. L. Am. J. Physiol. 81:36. 1927.
- 3. FRIEDMAN, S. M., JOHNSON, R. L., and FRIEDMAN, C. L. Circulation Research, 2:231. 1954.
- GLAUSER, K. F. and SELKURT, E. E. Am. J. Physiol. 168: 469. 1952.
 HANDLEY, C. A. and MOYER, J. H. J. Pharmacol. Exptl. Therap. 112: 1. 1954.
- OLIVER, J., MACDOWELL, M., and TRACY, A. J. Clin. Invest. 30: 1305. 1951.
 PHILLIPS, R. A., DOLE, V. P., HAMILTON, P. N., EMERSON, K., JR., ARCHIBALD, R. M., and VAN SLYKE, D. D. Am. J. Physiol. 145: 314. 1946.
- Selkurt, E. E. Measurement of renal blood flow. In Methods in medical research. Vol. I. Year Book Publishers, Inc., Chicago. 1948. p. 191.
- Vol. I. Year Book Publishers, Inc., Chicago. 1948. p. 191.
 9. Smith, H. The kidney: structure and function in health and disease. Oxford University Press, New York. 1951. p. 784.

EFFECT OF CORTISONE ON THE INTRACELLULAR DISTRIBUTION OF PHOSPHATASES AND RIBONUCLEASES IN RAT LIVER^{1, 2}

By Claude Allard, Gaston de Lamirande, George Weber,³
And Antonio Cantero

Abstract

The effect of cortisone acetate administration on the mitochondrial population and intracellular distribution of phosphatases and ribonucleases was investigated. Cortisone acetate was administrated at a dosage of 25 mgm. per 100 gm. weight, daily, during five days and sacrificed on the sixth day. The livers of these animals were removed and homogenized in 0.25 M sucrose and subjected to differential centrifugation to prepare nuclear, mitochondrial, microsomal, and supernatant fractions. Nuclear and mitochondrial counts were made for each homogenate prepared. From these the number of mitochondria per average cell was computed. The intracellular distribution of RNAase and PO4ase activities was determined and expressed per average cell and per liver/body weight ratio. The activity of the mitochondrial fraction was also expressed per average mitochondrion. Results of this investigation showed that cortisone lowered the number of large granules per liver cell without influencing the acid PO4ase and acid and alkaline RNAase activities per cell. This suggested that mitochondria isolated from cortisone treated rats possessed abnormally high acid PO4ase and RNAase activities or/and that the remaining population of granules was different from the one in liver cells of untreated animals. The investigation of alkaline phosphatase revealed that cortisone increased the Mg++ sensitive alkaline PO4ase localized in the supernatant fluid without influencing the Mg+ insensitive enzyme.

Introduction

Cortisone is known to induce important changes in the histology and biochemistry of rat liver (5, 8, 9, 11, 15). A recent report (8) suggested that large doses of cortisone caused a displacement of RNA from the mitochondria and microsomes into the submicrosomes. The possibility that cortisone induced a loss of RNA in the mitochondria or a loss of whole mitochondria has prompted us to study the effect of cortisone on the liver mitochondrial population. In view of previous findings which demonstrated that azo dye carcinogens decreased the number of mitochondria per average liver cell (1, 14), such a study is of interest. Since the mechanism by which liver cells are deprived of mitochondria during azo dye carcinogenesis is unknown, it seemed important to examine whether or not cortisone might have an effect similar to azo dyes.

The intracellular distribution of ribonucleases and of phosphatases have been investigated so as to provide additional information on the property of these enzymes in liver of cortisone treated rats. Acid and alkaline PO4ase activities have been previously studied in whole liver of cortisone treated rats

¹Manuscript received October 27, 1955.

Contribution from the Montreal Cancer Institute, Research Laboratories, Notre Dame Hospital, Montreal, Quebec. This investigation was supported in part by a grant-in-aid from the National Cancer Institute of Canada, and in part by an Institutional grant from the Defence Research Board of Canada (Grant 9310-53, Project D 50-93-10-53). One of us (G. de L.) held a Fellowship of the Damon Runyon Memorial Fund for Cancer Research, Inc., during this investigation.

²Abbreviations as follows: RNA, ribonucleic acid; RNAase, ribonuclease; POase, phosphatase.

³Senior Fellow of the Cancer Research Society.

by Kochachian *et al.* (7). They observed an increased alkaline PO₄ase activity *per unit wet weight* of liver in animals receiving cortisone, whereas acid PO₄ase was not affected (7).

Results of this study indicated that, per liver cell, the activities of the acid PO₄ase and of the acid and alkaline RNAase enzymes were not influenced by cortisone administration, whereas alkaline PO₄ase activity was increased.

The most striking observation at the intracellular level was the great activation exerted by cortisone on the average mitrochondrion enzyme activities. These "large granules" isolated from liver of cortisone treated animals showed about twice the acid PO4ase and acid RNAase activities of adequate controls.

Materials and Methods

The livers used in these studies were obtained from the same groups of animals sacrificed during the course of the experiments described previously (17). Four pools, each of four or five livers of young male Wistar rats (100 \pm 10 gm.), were employed in each group. The following experimental groups were set up.

(A) 1. Normal untreated fed rats.

2. Normal untreated 24 hr. fasted rats.

(B) 3. Vehicle injected rats, fed until sacrifice.

4. Cortisone injected rats, fed until sacrifice.

(C) 5. Vehicle injected rats, fasted 24 hr. before sacrifice.

6. Cortisone injected rats, fasted 24 hr. before sacrifice.

The animals were maintained on Purina Fox Chow and water ad libitum. The treated rats received daily intramuscular injections of 25 mgm. of cortisone acetate (Cortone, Merck) for five days and were sacrificed on the sixth day. The control animals were injected the same way with the vehicle, which contained only the suspending medium of cortisone, but no cortisone. The fasted animals were placed in individual cages with water available ad libitum.

Preparations of Tissue Homogenate and Cellular Fractions

The animals were stunned and decapitated, the liver was quickly excised, blotted on filter paper, put in a beaker, and chilled on cracked ice. As in previous studes, a pulp was prepared by forcing the pooled livers through a plastic squeezer (2). Connective tissue adhering to the sieves was discarded. An exact amount of tissue pulp was then homogenized in ice-cold 0.25 M sucrose to prepare a 10% suspension. Homogenization was carried out with a teflon plastic pestle turning at about 600 r.p.m. for two to three minutes. Less than one per cent of unbroken cells were present in the homogenate (2). The differential centrifugation method of Schneider and Hogeboom (13) was employed to prepare four cellular fractions: the nuclear (N), the mitochondrial (M), the microsomal (Mc) fractions, and the supernatant fluid (S). A refrigerated International Model PR-2 and a Spinco Model L centrifuge were used.

Each isolated fraction was diluted in $0.25\,M$ sucrose to the following concentration as compared to the original homogenate: (N), (M), (Mc): 20% and (S): 2.5%. The supernatant fluid contained the washings of all particulate fractions.

Quantitative Cytological Methods

Nuclear Counts

A 10% homogenate was prepared as described above, but in 0.85% saline. This medium permits a more uniform distribution of nuclei in the counting chamber than other media, such as sucrose. This has been established quantitatively.* An exact amount of saline homogenate was then measured with a drawn out serological pipette and transferred to a 10 ml. volumetric flask and completed to the mark with staining solution. The staining solution was prepared by dissolving 0.75 gm. of orcein in 200 ml. of a 45% acetic acid solution. A glass bead was then introduced in the flask which was agitated for one minute.

An aliquot of the homogenate was introduced in the hemacytometer by capillary attraction, and nuclei were counted. Twelve chambers were routinely counted per homogenate. Of this group of 12 counts, those which were out of the range by a factor of about 10–15% were repeated. The above mentioned method of diluting the homogenate for cell count was preferred to classical procedures involving more elaborate equipment (10). The advantage, besides its simplicity, is that the procedure for diluting the homogenate for the nuclear counts and for the biochemical work is the same. This appeared more accurate than by using one method of diluting the original homogenate for the cell count and a different one for the biochemical work.

Mitochondrial Counts

The "large granules" were counted in a Petroff-Hausser bacteria counter as previously described (3). Counts were done in the nuclear and mitochondrial fractions. Their sum was taken as the total number of mitochondria in the original homogenate (3).

Biochemical Procedure

The assay procedures for acid and alkaline PO_4 ases and RNAases were essentially the same as described previously (2, 4). Sodium- β -glycerophosphate (Eastman-Kodak, Rochester, N.Y.) and sodium nucleate (Schwarz Laboratories Inc., New York City) were employed as substrates for the PO_4 ase and RNAase studies respectively. The assay media were buffered with 2-amino-2-methyl-1,3-propanediol, and acetate-borate-cacodylate for alkaline PO_4 ases (2) and RNAases (4) respectively. Acetate – acetic acid buffer was employed for acid PO_4 ase (2). All activity measurements were carried out at 37° C. and at two levels of tissue concentration or in duplicates. Acid and alkaline PO_4 ase activities were measured in preincubated homogenate and fractions for reasons previously discussed (2).

^{*}Personal communication of Dr. Emma Shelton.

Results and Discussion*

Qualitative Cytological Data

Phase microscopic examination of liver cellular fractions isolated from sucrose homogenates revealed that vehicle and a 24 hr. fast, and especially cortisone, induced morphological alterations of the mitochondrial fraction. In the cortisone treated group, more than half of the cytoplasmic granules were greatly enlarged. These larger bodies were mostly, but not exclusively, found in the nuclear fraction; their diameter was about doubled; but no exact measurements were made. Furthermore, the "mitochondria" pellet isolated from the liver of cortisone and vehicle treated rats or from animals fasted for 24 hr. were always covered with a thin reddish and translucent layer. The presence of small particles in the mitochondrial fraction and the macroscopical appearance of the contaminating layer, described above, strongly suggested that microsomes were present in the mitochondrial fraction. Morphological alterations were not observed in the microsome fraction. The supernatant fluid was optically clear. The fractions prepared from normal liver of fed animals had the microscopical appearance repeatedly described in the literature (13).

Quantitative Cytological Data**

Cellularity

Table I shows that 24 hr. fasting produced a great increase in rat liver cellularity. These results were parallel with the known fact that the size of rat liver cell is decreased during fasting (6). On the other hand, fasting was without effect on liver cellularity of rats previously treated with the vehicle (Table I, third and fourth columns). The liver cellularity of vehicle treated animals (fed group) was the same as in the normal animal. Hence, the vehicle injection apparently prevented the increase in liver cellularity caused by fasting. This might be due to a cortisone release induced by the daily injection of the vehicle.

Confirming the results of Einhorn *et al.* (5) cortisone was observed to decrease the liver cellularity in animals normally fed. However, when the animals were fasted 24 hr. the decrease in cellularity was not so evident.

Mitochondriality

Table I shows that 24 hr. fasting or the injection of the vehicle had no effect on rat liver mitochondriality. On the other hand cortisone decreased the number of large granules per gram of tissue by about 50%.

Mitochondrial Population

The liver mitochondrial population of normal rat was decreased by 24 hr. fasting. These results parallel the decrease in the cytoplasm of the liver cell

* Reference is made of a previous paper where the effects of cortisone administration on the histology, liver body weight ratio, blood glucose level, and the glycogen content of these livers were reported (17).

** The term "cellularity" means the number of nuclei per unit wet weight of tissue (Ultman et al. (16)). In analogy the term "mitochondriality" is introduced here. "Mitochondriality" means the number of mitochondria or large granules per unit wet weight of tissue. "Mitochondrial Population" means the number of mitochondria or large granules per average cell (3).

TABLE I

QUANTITATIVE CYTOLOGICAL DATA* ON LIVER OF RATS FASTED DURING 24 HR.

AND OF RATS TREATED WITH CORTISONE

		Norma	al liver	Veh	icle	Cort	isone
	Variables	Fed	Fasted†	Fed‡	Fasted §	Fed‡	Fasted [
(A)	Cellularity∥ Nuclei/gm. wet weight × 10 ⁻⁶	261 ± 37	410 ± 54	275 ± 37	290 ± 43	209 ± 15	234 ± 38
(B)	Mitochondriality \parallel 1. Mitochondria/gm-equiv. of nuclear fraction \times 10 ⁻¹⁰	± 0.5	5.9 ± 0.7	6.2 ± 2.8	4.9 ± 2.0	± 0.5	3.8 ± 1.1
	2. Mitochondria/gm-equiv. of mito- chondrial fraction × 10 ⁻¹⁹	17.8 ± 3.4	18.0 ± 3.0	19.1 ± 2.5	18.0 ± 5.4	9.4 ± 1.1	± 2.7
	3. Sum	21.2	23.9	25.3	22.9	13.4	11.4
(C)	Mitochondrial population	830 ± 180	591 ± 102	994 ± 250	790 ± 137	641 ± 82	487 ± 90

- * Means and standard deviations of the means of four pools of four animals.
- † Fasted for 24 hr. Tap water ad libitum. † Daily injection for five days, food available till sacrificed on the sixth day.

Baily injection for five days, fosted 24 hr. before sacrificed on the sixth day.

See definition of terms in text foolnotes.

during fasting (6). In vehicle treated animals the effect of fasting was not so evident. On the other hand, cortisone decreased the mitochondrial population to a level comparable to fasting alone (24 hr.).

Thus the present study showed that 24 hr. fasting and cortisone administration lower the number of large granules per cell. Previous investigations in this (1) and another laboratory (14) demonstrated that carcinogenic azo dyes decreased the mitochondrial population of rat liver cells. The causes which underlie a loss of mitochondria or granules during cortisone treatment, however, might be different from the ones responsible during fasting or azo dye administration. Cortisone is known to increase the size of liver cell (8, 9) whereas fasting and azo dyes reduce it (6). A lower mitochondrial population in cells of increased size (as in cortisone treated rats) would indicate that the formation of cytoplasmic granules did not keep pace with the enlargement of the cell, and that their cytoplasmic concentration was lower. This mitochondrial decrease phenomenon in liver cells of cortisone treated animals might be related to the actual high glycogen content within these cells (17).

Enzyme Studies

Effect of 24 Hr. Fasting

Table II shows the effect of fasting on PO₄ase and RNAase activity per unit fresh weight of rat liver homogenate and per average liver cell and also the percentage distribution of activity in the various fractions isolated by differential centrifugation. Fasting (24 hr.) was found to increase the acid PO₄ase and acid RNAase activity per unit fresh weight, whereas it did not influence alkaline RNAase and alkaline PO₄ase activities. Per average cell, however (enzyme activity per unit fresh weight/number of nuclei per same unit fresh weight) RNAase and acid PO₄ase activities were not influenced by

ABLE II

EFFECT OF FASTING (24 HR.) ON VARIOUS ENZYME ACTIVITIES* OF RAI LIVER HOMOGENATE AND ON THE PERCENTAGE INTRACELLULAR DISTRIBUTION

		J. Ca	Acid	Y. C.	Acid	Alk	Alkaline		Alkaline	Alkaline PO, ases	
		Ď.	ase.	N.Y	.vase+	KN	. Jase &	+ Mg ⁺⁺	/g++	- Mg++¶	18++®
T	Fissue fractions	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
(A)	Activity per unit (H)	wet weig	ht of tissue	381	498	619	767	477	529	206	181
(B)	Activity per aver (H)	rage cell X	10-6	-1 -	1.24	2.41	1.89	18.4	13.3	H	H 4.
(C)	Percentage distri (N)	bution 9.3	F 1.5	16.9	18.6	14.4	15.2	15.0	14.5	38.3	
	(M)	51.0	+ + + + + + + + + + + + + + + + + + + +	50.0	+ 6:8+ + 6:8+ + 6:25	+ + 2.8	+ + + 3.2	+ +	+ + + + 0.4	16.4	12.7
	(Mc)	29.7	23.9	13.8	12.2	15.1	23.8	14.6	13.2	40.8	
	(S)	4.2	1 2.8	12.5	15.6	13.2	11.2	55.9	64.2	+ 2.6	
	Recovery	94.1	88.6	93.0	95.5	86.3	95.3	96.4	98.4	97.8	

* Means and standard deviations of the means of four pools of four rats.

4 Activity expressed as gamma of phosphorus liberated per hour from 0.08 M beta-glycerophosphate per 100 mgm, of fresh liver pulp at 37° and ‡ Activity expressed in arbitrary units (optical density corresponding to the acid soluble material liberated from RNA per gm. of liver pulp during final pH of 4.5 (Buffer: acetic acid - acetate) (2).

30 min. at 37° at final pH of 5.8 (Buffer: acctate-borate-cacodylate) (4). Activity expressed as for acid RN. lase - final pH 8.2.

Activity expressed as gamma phosphorus liberated from 0.1 M beta-glycerophosphate per hour per 100 mgm. of liver pulp at 37° in the presence of 0.01 M MgCl, at a final pH 9.2 (Buffer: 2-amino-2-methyl-1,3-propanediol) (2). Activity expressed as gamma phosphorus liberated from 0.1 M beta-glycerophosphate per hour per 100 mgm, of liver pulp at 37° in the absence of Mg ions at a final pH of 9.8 (Buffer: 2-amino-2-methyl-1,3-propanetiol) (2). fasting. The average liver cell in fasted rats showed a lower alkaline PO₄ase activity than in fed animals. In confirmation with the results of Rosenthal et al. (12), the Mg insensitive alkaline PO₄ase was affected by fasting. In fact, the fall in alkaline PO₄ase activities observed in the fasted animals was due to a decrease in PO₄ase activity as measured in the absence of Mg ions (Table II).

The percentage distribution of enzyme activities in the various liver fractions isolated by differential centrifugation was essentially the same in fasted and fed animals. This indicates that the changes in activity described for the whole homogenate or average cell occurred proportionally in all cellular fractions. The alkaline PO4ase activity of liver cellular fractions isolated from fed animals appears to vary greatly from one animal to another (as illustrated by the great S. D.; Table II, second to the last column). This was due to one abnormally low determination in this series.

The activities of RNAase and PO₄ase expressed per liver/body weight ratio of normal liver of fed and fasted rats are given in the second and third columns of Table IV. These data are in accordance with those expressed on a per cell basis. Acid PO₄ase and acid and alkaline RNAase activities per liver/body weight ratio were not changed by the 24 hr. fasting, whereas less alkaline PO₄ase activity in liver, as measured in the absence of Mg⁺⁺, was "available" to fasted than to fed rats.

Effect of Cortisone

Table III shows the effect of cortisone administration* on RNAase and PO4ase activities per unit fresh weight and per average cell and also the percentage distribution of activities in the (N), (M), (Mc) fractions and in the (S) fluid.

On fresh weight basis, rat liver acid PO₄ase, acid RNAase, alkaline PO₄ase (as measured in the absence of Mg) activities appeared to be inhibited by cortisone. This inhibitory effect of cortisone per fresh weight of liver was evident in the fed and fasted groups, except for alkaline RNAase activity which was not changed when the cortisone treatment was followed by a 24 hr. fast.

On a per cell basis, an activation of the Mg sensitive alkaline PO₄ase was observed following cortisone injection. This activation was evident, however, only in normally fed animals. It has been shown that fasting alone decreased the Mg insensitive alkaline PO₄ase activity (Tables II and IV) without influencing the Mg sensitive enzyme. In cortisone treated rats, fasting did not provoke the same phenomenon. The general picture which emerged from this is confused but apparently cortisone would be an activator of the Mg sensitive PO₄ase, whereas fasting would lower the Mg insensitive alkaline PO₄ase activity (see Table IV). This is further evidence that rat liver contains two distinct alkaline PO₄ases (2, 12).

^{*}Cortisone did not alter the PO4ase and RNAase activities of rat liver homogenate in vitro.

ABLE III

EFFECT OF CORTISONE ON VARIOUS ENZYME ACTIVITIES* OF RAT LIVER HOMOGENATE AND ON THE PERCENTAGE INTRACELLULAR DISTRIBUTION

			A. P.	Acid	7.0	Acid	All	Alkaline		Alkaline	Alkaline Potases	
				430	N.N	200	ė.	7435	+	+ Mg**		Mg+
	Tissue fractions	Treatment	Fed:	Fasted §	Fed:	Fasted	Fed:	Fasted	Fed:	Fasted	Fed:	Fasted
(A)	Activity per unit wet weight of tissue H	tht of tissue Vehicle		1366	434	530	919	880	57.2	550	210	264
		Cortisone	+ 118 825 + 70	+ 907 + 69	278	+ + 44 44	± 451 + 24	+ 1033 + 376	± 100 ± 638 ± 17	± 513	150	+ 14 80
8	Activity per average cell >	× 10-6 Vehicle	37	47			2			19	7.7	
		Cortisone	# H # 39.7	4 4 30.8 5.0 5.0	+ 0.34	+ 0.38	+ 10.06	+ 4.56 + 2.10	± 30.6 ± 1.7	+ 22.4	+ + 7.1	+ +
0	Percentage distribution	Vehicle	9.1	2		-	15.2		13.1	1	=	1
		Cortisone	+ + + 9.4 + 5.1	+ + + 11.2	15.8	± 18.50 ± 18.5	+ + 16.6 16.16	H H 3.2	± 2.0 ± 12.7	1	+ + + 0.0 0 - 0.0	1
	M	Vehicle	51.3	49.3	47.8	39.5	42.9	33	6.3	1	9	1
		Cortisone	4 14 14	+ + 80.1 8.3	43.5	H 47.8	+ + 53.2 5.3.3	H H 6,00,00 8,00,00	H H		+ + + 5.5	1
	Mc	Vehicle	29	27	*	14.5	27.	24	10	1		1
		Cortisone	± 22.8	# 22.7 # 1.8	H H	H H 5.1	H 133.3	± 22.5	H H	1	+ 2.0	1
	S	Vehicle		0.	*	5.	12.4	12	51.9	-	17.4	1
		Cortisone	+ 1 5.3	10.6	13.4	16.8	± 10.0	H +1	# # 4.7	ı	32.0	I
	Recovery	Vehicle	94.1	6.46	98.2		98.8	87.1		I	112.2	1
		Cortisone	98.2	105.2	84.2	94.2	92.7	85.0	107.3	1	128.1	î

* As defined in Table II.

+ Mean's and standard deviations of the means of four pools of four animals.

Daily injection for five days, food available till sacrificed on the sixth day.

Daily injection for five days, fasted 24 hr. before sacrificed on the sixth day.

TABLE IV ENZYME ACTIVITIES* PER LIVER/BODY WEIGHT RATIOT X 100

	Norm	al liver	Ve	hicle	Cor	tisone
Enzymes	Fed‡	Fasted‡	Fed:	Fasted‡	Fed‡	Fasted!
Acid PO ₄ ase	4440 ± 532	5018 ± 837	4840 ± 444	5751 ± 641	6406 ± 607	6248 ± 762
Acid RNAase	2055 ± 158	1829 ± 107	1999 ± 384	2285 ± 233	2166 ± 302	2238 ± 303
Alkaline RNAase	3199 ± 534	2810 ± 113	$^{2843}_{\pm\ 223}$	3718 ± 391	3518 ± 454	6970 ± 2070
Alkaline PO ase (A) + Mg -	2340 ± 213	$^{2023}_{\pm\ 127}$	2807 ± 576	2335 ± 547	4963 ± 415	3589 ±1000
(B) - Mg++	1013 ± 165	679 ± 142	995 ± 85	1131 ± 452	1649 ± 308	997 ± 645

^{*} As defined in Table II; means and standard deviations of the means of four pools of four animals.

† Given in the previous paper (17). ‡ See footnote Table I.

At the intracellular level some changes in the activity of RNAase and PO4ase were evident. The most important among these were a loss of acid PO4ase and of acid and alkaline RNAase activity in the microsome fraction (as reflected in Table III, by a lower percentage of activity in the microsome fraction of cortisone as compared to vehicle treated rats). These lower values in the microsome fraction were somewhat compensated by increase of activity in other fractions. The supernatant fluid of cortisone treated rats (fasted for 24 hr.) contained more acid PO4ase activity whereas the mictochondria contained more RNAase activity than did the vehicle controls.

Table IV shows the effect of cortisone on RNAase and PO4ase activity, as expressed per liver/body weight ratio. An increase in acid PO4ase (in fed animals only), alkaline RNAase, and alkaline PO₄ase (mostly the Mg⁺⁺ sensitive) was evident in animals receiving cortisone. Hence, these liver enzyme activities would appear more "available" to animals subjected to cortisone treatment.

It is of importance to note that cortisone, which did not influence acid PO4ase and RNAase activities per liver cell, decreased the number of large granules per cell (Table I). This is of interest because a high proportion of the activity of these enzymes is apparently linked to granules present in this fraction (2, 14). To illustrate this, the activities of acid PO4ase and RNAase per average mitochondrion were calculated (3). The mean activity in the mitochondrial fraction was divided by the corresponding mean number of granules counted in this fraction. Table V demonstrates that the average mitochondrion isolated from liver of cortisone treated animals (either fed or fasted 24 hr. at time of sacrifice) showed a great increase of acid PO4ase, acid RNAase, and alkaline RNAase activities as compared to the controls (except for acid RNAase activity of the average liver mitochondrion of cortisone treated rats fed continuously, which remained constant).

TABLE V

ENZYME ACTIVITIES OF THE AVERAGE MITOCHONDRION ISOLATED FROM LIVER HOMOGENATE OF RATS TREATED WITH CORTISONE

		Activity per mitoc	chondrion *× 1010	0
	F	ed†	Fas	sted‡
Enzymes	Vehicle	Cortisone	Vehicle	Cortisone
Acid PO ₄ ase	274	507	373	717
Acid RNAase	10.7	12.8	11.6	20.5
Alkaline RNAase	13.9	25.5	16.5	49.6

* Activity per gm-equivalent of fresh mitochondrial fraction/number of mitochondria per gm-equivalent of mitochondrial fraction.

Daily injection for five days, food available till animals sacrificed on the sixth day.

Daily injection for five days, fasted 24 hr. before sacrificed on the sixth day.

The average mitochondrion isolated from liver of fasted rats (24 hr.) showed similarly a 50% increase in acid PO4ase and 25% increase in RNAase activities as compared to the average mitochondrion isolated from fed animals.

These results would imply that the granules remaining in rat liver cells after cortisone treatment or fasting were either richer in acid PO4ase and RNAases or/and that the remaining population of granules was different from the one in liver cells of untreated animals. The significance of these changes in enzyme activity in the large granule fraction is far from being understood. Further studies with more refined centrifugal technics are conducted.

Acknowledgment

The authors are indebted to Miss Denise Plante and Mr. Jean Zytko for technical assistance. The authors are grateful to Dr. J. H. Laurie of Merck Co. of Montreal for a gift of Cortisone Acetate (Cortone).

References

- Allard, C., de Lamirande, G., and Cantero, A. Can. J. Med. Sci. 30: 543. 1952.
 Allard, C., de Lamirande, G., Faria, H., and Cantero, A. Can. J. Biochem. Physiol.
- 32: 383, 1954.
 3. ALLARD, C., MATHIEU, R., DE LAMIRANDE, G., and CANTERO, A. Cancer Research, 12: 407, 1952.
- 12: 407. 1952.
 4. DE LAMIRANDE, G., ALLARD, C., and CANTERO, A. Science, 119: 351. 1954.
 5. EINHORN, S. L., HIRSCHBERG, E., and GELLHORN, A. J. Gen. Physiol. 37: 559. 1954.
 6. HARRISON, M. F. Biochem. J. 55: 204. 1953.
 7. KOCHANIAN, C. D. and BARTLETT, M. N. J. Biol. Chem. 176: 243. 1948.
 8. LOWE, C. U. and WILLIAMS, L. W. Proc. Soc. Exptl. Biol. Med. 84: 70. 1953.
 9. PEREZ-TAMAYO, R., MURPHY, W., and IHNEN, M. Arch. Pathol. 56: 629. 1953.
 10. PRICE, J. M. and LAIRD, A. K. Cancer Research, 10: 650. 1950.
 11. ROBERTS, K. B., FLOREY, H. W., and JOKLIK, W. K. Quart. J. Exptl. Physiol. 37: 239.

- 12. Rosenthal, O. and Vars, H. M. Proc. Soc. Exptl. Biol. Med. 86: 555. 1954.
 13. Schneider, W. C. and Нодевоом, G. H. J. Biol. Chem. 183: 123. 1950.
 14. Striebich, M. J., Sbelton, E., and Schneider, W. C. Cancer Research, 13: 279. 1953.
 15. Тімікаs, P. S. and Косн, P. Anat. Record, 113: 349. 1952.
 16. Ultman, J. E., Hirschberg, E., and Gellhorn, A. Cancer Research, 13: 14. 1953.
 17. Weber, G., Allard, C., de Lamirande, G., and Cantero, A. Endocrinology. 1956. In press.

ACETATE METABOLISM OF MATURING WHEAT PLANTS¹

By W. B. McConnell and L. K. Ramachandran²

Abstract

The transport of carbon-14 injected into the hollow stems of growing wheat plants in the form of sodium acetate-1-C¹⁴ and -2-C¹⁴ was studied. The labelling efficiency of the tracer and its distribution among components of the wheat kernels was markedly dependent upon the time of injection. Maximum incorporation of activity occurred with plants which were given the tracer about 80 days after seeding. Sodium acetate-1-C¹⁴ was less effective for producing labelled kernels and gave rise to more uniform distribution of carbon-14 among the components, very little carbon-14 being utilized for starch synthesis nearer maturity. A high percentage of the carbon-14 content of the gluten resided in the glutamic acid residues. Glutamic acid-C¹⁴ injected into the stems was an efficient source of labelling for the plant. The results are consistent with the view that acetate is utilized by way of the Krebs' citric acid cycle.

Introduction

Radioactive tracers are being widely used to study specific reactions occurring in plants (8, 10), and have proved very useful for experiments on plant metabolism and plant biosynthesis (7, 9). A number of experiments have been reported on the absorption and utilization of carbon-14-labelled compounds by wheat plants (9) in short-term experiments. However, information is lacking about the incorporation of such compounds into the various parts of the plant and into the major chemical constituents of wheat plants that have been exposed to the labelled compounds for prolonged periods.

This communication reports a study of the uptake of sodium acetate-1-C¹⁴ and -2-C¹⁴ by wheat plants. The objective was to investigate the utilization of these compounds by maturing wheat plants and in particular to determine whether wheat gluten labelled with carbon-14 could be obtained by injecting labelled acetate into the stem of the plants. It was expected that gluten of appreciable activity could be obtained only if the isotope was administered during a stage of active growth and metabolism and if the plant subsequently was allowed to ripen fully, thus providing seed proteins typical of mature wheat.

Experimental Methods

Growth Conditions for Wheat

Thatcher wheat was seeded May 29 in an open field plot. Weather conditions were unusually moist and cool throughout the growing season (1954), and, although the plants grew well, the grain was somewhat slow in developing and late in maturing. Wind and heavy rains caused some lodging of the grain in September. Leaf rust appeared on the grain in early July and this, together with stem rust, reduced the quality of the grain.

¹Manuscript received October 31, 1955.

Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Issued as N.R.C. No. 3847. Presented in part at the Conference on Radioactive Isotopes in Agriculture at Michigan State University, East Lansing, Michigan, January 1956.

² National Research Council of Canada Postdoctorate Fellow 1953-55.

The plants containing C¹⁴-labelled compounds were harvested on September 20 at which time growth and development had apparently ceased. The grain and chaff were collected, dried, and ground in a micro-Wiley mill to pass a 40 mesh screen.

Determination of Specific Activity of Samples

All determinations of carbon-14 were done by a wet combustion method in which the carbon from the samples was converted to carbon dioxide and counted as a gas (2). The specific activity is expressed as millimicrocuries per millimole of carbon dioxide ($m\mu c./mM$. CO_2).

Administration of Radioactive Acetate to Wheat Plants

Sodium acetate, C¹⁴ labelled, was injected into the hollow stems of selected plants (1) with a clinical syringe. An aqueous solution of the compound (0.2 ml. containing 5 µc. of C¹⁴) was administered to each plant at a point about two inches below the top node of the stem. A syringe needle was inserted low in the same internode to permit air displaced by the solution to escape. Collodion solution was brushed over the perforations in the stem to seal them. To six tillers, one each from plants at six places in the plot, was administered sodium acetate-1-C¹⁴ on the same day and the injections were repeated at several stages of growth using tillers from different plants. Sodium acetate-2-C¹⁴ was administered to other plants at the same times and according to the same scheme. The products from all suitable plants recovered for the same isotope and day of injection were combined. Many plants did not develop properly or were lost entirely because of adverse weather conditions.

A summary of the conditions for obtaining the labelled wheat plants is given in Table I.

Separation of Wheat into Components

The scheme outlined in Fig. 1 was used to separate the wheat meals into their major chemical components. A rigid time schedule was adopted for the separation, and the same apparatus and equipment was used for all samples in an effort to provide the best possible comparison between products from different meals. Comments on the various steps follow.

Ether Extraction

One gram of meal was extracted with 50 ml. of ether for $15\frac{1}{2}$ hr. in a Soxhlet apparatus and the residue was air-dried. The extract was evaporated to dryness, and the residue was taken up in 4 ml. of ether and extracted twice by shaking with 4 ml. portions of water. The ether layer was evaporated to dryness and after the weight of extracted lipid was determined 4 ml. of ether was added and the solution was stored in sealed glass bottles.

Salt Extraction

The defatted meal was stirred vigorously for one hour with 15 ml. of 5% K_2SO_4 (6). The residue was collected by centrifugation and re-extracted with 10 ml. of 5% K_2SO_4 . The combined supernatant solution was stirred

TABLE I
GROWTH CONDITIONS OF LABELLED WHEAT SAMPLES
(Thatcher wheat—sown May 29; harvested September 20, 1954)

		Growth ti	Growth time, days		Weight recovered, gm.	vered, gm.
Date C ¹⁴ administered	Material administered	Before inoculation	After	No. of heads recovered	Chaff	Wheat
July 30	Sodium acetate-2-C ¹⁴	09	55	85	0.78	1.98
August 9	99	73	42	2	0.49	1.17
	99	77	38	83	69.0	1.59
August 30	77	94	21	5	1.02	2.34
September 3	25	86	17	4	99.0	2.08
	Sodium acetate-1-C14	09	55	2	0.34	0.98
August 9	99	73	42	2	0.44	0.53
August 13	99	77	38	2	0.43	1.14
August 30	10	94	21	9	1.10	2.77
September 3	33	86	17	4	1 07	2 44

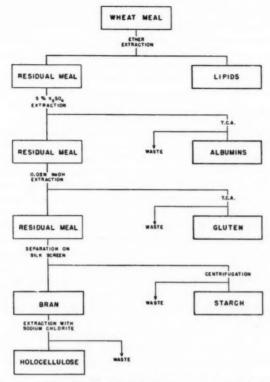


Fig. 1. Scheme for separation of components from wheat meal.

gently for one-half hour with 10 ml. of 10% trichloracetic acid (TCA). The precipitated protein designated as the "albumin" was collected by centrifugation, washed with acetone and then with ether, and was dried under vacuum overnight.

Alkali Extraction

The meal, after salt extraction, was stirred first with 15 ml. and then with 10 ml. of 0.02 N sodium hydroxide, and the protein was isolated and dried as described above for albumin. The alkali soluble protein was designated "gluten" and possessed the characteristic elastic properties of this protein.

Isolation of Starch

The residue from the protein extractions was suspended in 25 ml. water, filtered through a 10 XX silk screen, and washed on the screen with an additional 75 ml. of water. The starch in the filtrate was collected by centrifugation, and was washed first with acetone and then with ether, and dried overnight under vacuum.

Collection of Bran

The bran fraction, which remained on the screen after the starch had been washed through, was dried by washing with acetone and ether and stored under vacuum overnight. Holocellulose was prepared from the bran by extraction with sodium chlorite (5).

Experimental Results

Incorporation of C14 Into Wheat Kernels and Chaff

Table II contains data on the distribution of activity in the kernels and in the chaff of plants labelled with acetate. An optimum time for injection was expected because administration at an early stage would increase the probability of loss through the respiration process as carbon passed through successive cycles of plant metabolism. On the other hand, administration near the time of maturity when plant growth is slow would not afford opportunity for incorporation. The results indicate that appreciable activity was present in the heads of the wheat, the amount retained at maturity being markedly dependent upon the age of the plant at injection. The data also indicate that maximum incorporation of the tracer into the wheat kernels occurs when injections are made at an age of about 80 days. The maximum is most pronounced with sodium acetate-2-C¹⁴, the compound which also gave the most active sample. In the most active of the wheat samples labelled with sodium acetate-2-C¹⁴ 37% of the carbon-14 administered is accounted for in the heads of the plants.

The ratio of the specific activity of the chaff to that of the wheat increases with the age at which the injection is made. This suggests that metabolic activity in the chaff continues at a later stage of development. Sodium

TABLE II

ACTIVITY OF WHEAT AND CHAFF FROM LABELLED WHEAT PLANTS

Age at administration, — days	Activity recovered, μc. C ¹⁴ per plant		Specific activity, mμc./mM. CO ₂	
	Wheat	Chaff	Wheat	Chaff
	Plants admir	nistered sodium ace	etate-2-C14	
60	0.827	0.107	36.4	11.6
	1.27	0.099	61.6	12.1
73 77	1.66	0.222	90.4	29.2
94	0.464	0.382	27.8	57.4
98	0.304	0.349	16.4	64.3
	Plants admi	nistered sodium acc	etate-1-C14	
60	0.683	0.070	39.6	12.7
73	0.054	0.073	5.8	10.6
77	0.866	0.125	43.7	16.9
94	0.653	0.180	41.8	25.0
98	0.244	0.362	11.8	41.8

acetate-2-C¹⁴ in general promotes greater activity in the chaff than does sodium acetate-1-C¹⁴. This is in agreement with the observations made on the wheat kernels and may occur, at least in part, because the latter is more readily converted to C¹⁴O₂ by metabolic processes.

Distribution of C14 Among Chemical Components of the Wheat Kernels

Table III contains data indicating the yields of the different components recovered when the wheat kernels were fractionated as described above. About 93% of the sample weights were accounted for in the products listed, the remaining material is assumed to have been lost in the various waste solutions indicated in the chart (Fig. 1). The specific activity of the components is shown in Table IV. In general the protein is the most active material in the wheat kernel. All gluten samples and most of the preparations designated "albumin" have greater specific activity than the corresponding wheat meals. The preferential incorporation of acetate-carbon into the protein is particularly evident during later stages of growth. Thus the ratio of gluten specific activity to wheat specific activity for samples from plants fed sodium acetate-2-C¹⁴ at ages of 77, 94, and 98 days are 2.14, 2.44, and 1.77 respectively, while the same ratios for plants fed sodium acetate-1-C¹⁴, are 1.05, 2.09, and 2.45 respectively.

The incorporation of C¹⁴ into the starch decreases markedly during later stages of growth and after the 98th day only a very small amount is taken up by the starch. Since purification of the starch by iodine precipitation (3) failed to alter appreciably the activity, the active carbon appears to be an integral part of the starch molecule. Fig. 2 shows the ratio of starch activity to gluten activity and illustrates the dependence of C¹⁴ distribution upon time of injection. The percentage of the activity of the wheat kernels recovered by the separation scheme used was shown (Table IV) to decrease with the age of the plants at injection. Since the activity lost must be assumed to have been discarded with various waste extracts, the results indicate that with shorter times of exposure to the tracer a larger proportion of the activity is present in the form of water soluble material.

TABLE III

RECOVERY OF WHEAT COMPONENTS FROM 1 GM. OF WHEAT MEAL

Component	Average weight recovered, gm.	Average deviation from mean, gm.
Lipid	.0294	.0061
Albumin	.0390	:0041
Gluten	.0799	.0027
Starch	.5593	.0145
Bran	. 1469	.0114
Total	.8545	.0388
Moisture	.0778	.0158
Total accounted for:	.9323	

TABLE IV
DISTRIBUTION OF ACTIVITY AMONG WHEAT COMPONENTS

			Specific a	Specific activity, mµc./mM. CO ₂	M. CO ₂			, , ,
Age at administration	Wheat meal	Lipid	Albumin	Gluten	Starch	Bran	Holo- cellulose	% of meal activity recovered
			Sodi	Sodium acetate-2-C14	*			
09	36.4	23.4	53.2	55.2	20.6	28.9	28.8	71.0
73	61.6	39.9	62.2	8.68	51.4	31.3	32.4	8.02
1.	90.4	97.2	93.9	201.5	50.1	51.0	40.1	70.5
14	27.8	25.5	118.7	67.7	2.6	31.3	15.4	62.8
80	16.4	11.8	53.6	29.0	0.3	13.5	8.6	38.3
			Sodi	Sodium acetate-1-C14	•			
0	39.6	21.2	43.1	49.1	33.0	35.9	34.1	78.5
3	80.00	2.4	3.2	7.4	4.5	3.3	2.5	70.8
1	43.7	26.7	39.6	45.9	32.2	28.2	24.3	67.7
94	41.8	32.2	80.1	87.4	10.2	33.7	30.2	66.2
80	11.8	15.2	39.4	28.9	1.1	14.0	00	63.5

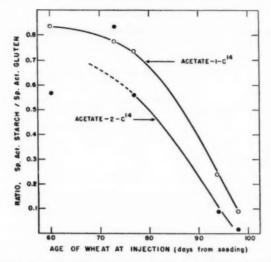


Fig. 2. Relation between the age of wheat plants when sodium acetate was administered and the ratio of the specific activity of starch to specific activity of gluten.

Some differences between sodium acetate-1-C¹⁴ and -2-C¹⁴ labelled wheats are noted. As mentioned above, acetate-2-C¹⁴ is in general incorporated to a greater extent than the carboxyl-labelled acetate. The distribution of activity among the wheat components is also somewhat more variable for plants labelled with sodium acetate-2-C¹⁴ than with sodium acetate-1-C¹⁴.

Some simple calculations were done to estimate this non-uniformity in an approximate way. The arithmetical differences between the specific activity of the several constituents from that of the corresponding wheat kernels were taken. The averages of the differences for each wheat meal were calculated and expressed as percentage of the specific activity of the meal. A comparison of the results for the sodium acetate-1-C¹⁴ with those for sodium acetate-2-C¹⁴ is given in Table V. It indicates that for all times of injection recorded the sodium acetate-2-C¹⁴ causes the widest variation in the amounts of carbon-14 among the components. The results suggest that carbon-1 of sodium acetate enters into more of the synthetic processes of the plant than does carbon-2.

Isolation of Glutamic Acid from Labelled Wheat Kernels

In a preliminary experiment glutamic acid was isolated from two samples of labelled gluten. The gluten was hydrolyzed for six hours in sealed tubes with hydrochloric acid at 120° C. The hydrolyzate was decolorized with charcoal, concentrated, and the glutamic acid hydrochloride which crystallized out upon standing in the cold was collected and recrystallized from concentrated hydrochloric acid. Glutamic acid from wheat fed sodium acetate-2-C¹⁴ at the age of 73 days possessed a specific activity of 235 mµc./mM. CO₂ as

TABLE V

Variation of specific activity of wheat kernel components from that of corresponding kernels for plants fed sodium acetate-1-C14 and -2-C14

	Average % difference components from	e of activity of plant that of kernels*
Age at administration	Sodium acetate-1-C14	Sodium acetate-2-C14
60	21.5	39.6
77	24.8	58.0
94	55.8	116.5
98	89.0	97.0
Average difference	47.8	77.5

^{*} Percentage differences of activity of components (lipid, albumin, gluten, starch, and cellulose) from that of meal are averaged. Sample injected at age of 73 days not included in comparison because of the "abnormally" low activity obtained with sample given sodium acetate-I-C14 at this date.

compared with a specific activity of 89.8 for the gluten from which it was derived. Glutamic acid from wheat labelled with sodium acetate-1-C14 at an age of 94 days had an activity of 327 m μ c./mM. CO2 as compared with the gluten of activity 87.4 m μ c./mM. CO2. From this striking increase in activity it is evident that a large part of the protein activity resides in the glutamic acid.

Experiments with Uniformly Labelled Glutamic Acid

Thatcher wheat plants were grown indoors in gravel culture using Hoaglund's solution No. 2, iron being supplied as the ethylene diamine tetraacetate (4). Artificial light was supplied to the plants at an intensity of from 800–1000 ft-c. for 18 hr. per day. During most of the growing period the temperature of the room was $22^{\circ}-25^{\circ}$ C. Seventy-three days after seeding 0.2 ml. of solution containing 5 μ c. of glutamic acid-C¹⁴ was injected into each of five plants. The method of administering tracer was as already described except that it was given above the top nodes of the plants. When injected, the plants were growing vigorously and were in blossom, but shortly thereafter the temperature in the room unavoidably rose above normal and caused rapid maturation of the plants (harvested on 88th day). The resultant grain was somewhat shrunken and was of poor quality.

Like parts of the plants were combined and their radioactivity measured. The results (Table VI) show that about 50% of the activity was lost from the plants, but indicate, nevertheless, that wheat plants retaining appreciable activity can be obtained by administering glutamic acid-C¹⁴ in the hollow stem. Since some activity was detected in young plants to which no tracer was given it is suggested that at least some of the activity observed below the site of injection and in the leaves may have been acquired by reabsorption of labelled CO_2 .

TABLE VI

RECOVERY OF RADIOACTIVE CARBON ADMINISTERED TO WHEAT PLANTS AS I-GLUTAMIC ACID

	Weight collected, gm.	Specific activity, mµc./mM. CO ₂	% of C ¹⁴ administered
Grain	3.64	65.7	25.2
Chaff	1.55	62.9	10.9
Stem above top node	1.73	52.3	10.0
Stem lower parts	3.20	0.95	0.3
Leaves (for entire plant)	4.63	1.98	1.0
Remainder of plant parts	_		0.2
Total recovery		_	47.6

TABLE VII

DISTRIBUTION OF ACTIVITY IN FRACTIONS OF WHEAT INJECTED WITH I-GLUTAMIC ACID-C16

Constituent	Yield, % of wheat	Specific activity, mµc./mM. CO ₂	Specific activity, mµc./mgm.	of activity in wheat kernels recovered
Meal	(100)	65.7	2.37	(100)
Albumin	4.6	158.0	4.94	9.6
Gluten	15.9	165.1	6.95	46.7
Starch	51.2	13.1	0.45	9.7
Bran	12.7	38.2	1.35	7.3
Total	84.4			73.3

The wheat was separated into fractions as previously described. The results (Table VII) are consistent with the view that some of the labelled glutamic acid may have been incorporated into the protein.

Conclusions

It is concluded that mature wheat retaining a satisfactory yield of carbon-14 can be obtained by injecting the tracer in the form of sodium acetate into the hollow stem of the growing plants, and that, for the conditions used, injection at about 80 days after seeding gives the highest specific acitivity in the grain. Injection at later stages favors incorporation of the tracer into the protein with very little activity appearing in the starch. The methyl-carbon of the acetate in general is incorporated more efficiently than the carboxyl-carbon and gives the most variation in distribution of carbon-14 among the components. The results are consistent with those expected if the acetate were utilized largely via the Krebs' citric acid cycle, perhaps with α -keto glutaric acid being preferentially withdrawn from the cycle at some stages of development for glutamic acid and protein synthesis.

Acknowledgment

The authors are indebted to Dr. A. C. Neish for valuable advice and assistance throughout the work and for growing the wheat plants injected with glutamic acid-C14. The technical assistance of Mr. John Dyck, Mr. A. Rose, and Mr. M. Mazurek is acknowledged.

References

- 1. Brown, S. A. and Neish, A. C. Can. J. Biochem. Physiol. 32:170. 1954.
- 2. Buchanan, D. L. and Nakao, A. J. Am. Chem. Soc. 74: 2389. 1952.
- 3. Calvin, M., Heidelberger, C., Reid, J. C., Tolberg, B. M., and Yankwich, P. F. Isotopic carbon. John Wiley & Sons, Inc., New York. 1949. p. 266.
- JACOBSON, L. Plant Physiol. 26: 411. 1951.
 JERMYN, M. A. Modern methods of plant analysis. Edited by K. Paech and M. V. Tracey. Verlag von Julius Springer, Berlin. 1955. p. 203.
- 6. LEPPER, H. A. Editor. Official and tentative methods of analysis of the association of official agricultural chemists. 6th ed. Association of Official Agricultural Chemists, Washington, D.C. 1945. p. 286.
- 7. Neish, A. C. Can. J. Biochem. Physiol. 33:658. 1955.
- 8. Newburgh, R. W. and Burris, R. H. Arch Biochem. and Biophys. 49:98. 1954.
- 9. VITTORIO, P. V., KROTKOV, G., and REED, G. B. Can. J. Botany, 33: 275. 1955.
- 10. WILSON, D. G., KING, K. W., and BURRIS, R. H. J. Biol. Chem. 208: 863. 1954.

THE CONSTITUENTS OF COD LIVER WITH VITAMIN B₁₂ ACTIVITY FOR LACTOBACILLUS LEICHMANNII¹

BY BERYL TRUSCOTT AND P. L. HOOGLAND

Abstract

The identity of the compounds that together form the total microbiological vitamin B_{12} activity of cod-liver residue was determined by a fractionation procedure involving ion exchange, countercurrent distribution, partition chromatography, and microbiological assay. The presence of cyanocobalamin, hydroxocobalamin, and the desoxyribosides of thymine, uracil, hypoxanthine, xanthine, and guanine was demonstrated. The total vitamin B_{12} activity, as determined by microbiological assay with Lactobacillus leichmannii, and its distribution in cod-liver residue, were very similar to those of beef liver. Codliver residues may be used to replace beef liver in vitamin B_{12} preparations.

A procedure has been developed in this laboratory (12) for the production of cod-liver residue which is relatively free of oil but contains large amounts of protein and B-vitamins (6, 11). The high vitamin B_{12} activity of the material seemed to make it particularly interesting to the pharmaceutical and feed manufacturing industries.

For a more complete evaluation of the nutritive properties of the residue, it was thought necessary to compare with data available for beef liver the "total vitamin B_{12} activity", as determined by microbiological assay, and the qualitative composition of the mixture of constituents that together form this total activity.

Experimental

1. Raw Material

The raw material used in this work was dried, defatted cod-liver residue (11).

2. Equipment and Methods

Ion Exchange

Columns of Amberlite IRA-400* in the hydroxyl form were used; they were operated under constant pressure. The eluant was 0.05 molar acetic acid.

Countercurrent Distribution

The 25-tube apparatus described by Craig and Post (2, 3) was used; the solvent system was isobutanol/water/acetic acid.

Partition Chromatography

Columns of purified potato starch (4) were used with n-butanol/water.

Paper chromatography was done on Whatman No. 1 paper strips. Various solvent systems were used: n-butanol/water, n-butanol/water/ammonia, n-butanol/water/ethyl acetate/morpholine, and aqueous disodium hydrogen phosphate/isoamyl alcohol. The positions of growth factors on the paper

*The Rohm and Haas Company, Washington Square, Philadelphia.

¹Manuscript received in original form October 11, 1955, and as revised, December 2, 1955. Contribution from the Fisheries Research Board of Canada, Halifax, Nova Scotia.

were determined by bio-autography according to Winsten and Eigen (13); the medium was Difco-CS vitamin B₁₂ agar, the organism *Lactobacillus leichmannii* (A.T.C.C. 4797).

Absorption Spectra

Ultraviolet absorption spectra were determined with the Beckman DU spectrophotometer; the instrument was equipped with standard 10 mm. cells, as well as with a microattachment* and an adapter for direct photometry of paper strip chromatograms (10).

Microbiological Assay of Vitamin B₁₂ Activity

The procedure and medium described by Peeler et al. (9) were used.

3. Results

Five hundred grams of dried, defatted liver residue was extracted exhaustively with water; the extract concentrated in vacuo to 125 ml. Microbiological assay before and after treatment with alkali to destroy cobalamins showed that at least 75% of the total activity was due to these compounds and the remainder to alkali-stable growth factors. The total vitamin B₁₂ activity was 1.5 µgm. per gm. The concentrate was extracted with isobutanol, the solvent removed from the extract by vacuum distillation, and the residue taken up in 100 ml. of water. The extract was dark brown and had a solid content of 12.6%. It was shown by paper chromatography that the aqueous concentrate contained at least four and probably six growth factors with vitamin B₁₂ activity, and that the non-vitamin B₁₂ part had been extracted practically quantitatively by the isobutanol whereas the vitamin B₁₂ remained in the aqueous layer. The isobutanolic extract contained at least three growth factors. The aqueous layer was subjected to chromatography on phosphate-treated paper (14) and the presence of cyanocobalamin (vitamin B₁₂) and hydroxocobalamin (vitamin B_{12a}) was demonstrated.

The extract obtained with isobutanol was purified by passage through a column of Amberlite IRA-400 and elution with 0.05 molar acetic acid. This treatment removed 59% of the solids but none of the vitamin B₁₂ activity. The resulting solution was evaporated to dryness and the residue taken up in 25 ml. of "bottom solvent" (isobutanol/water/acetic acid). Portions of 8 ml. were then subjected to 25-stage countercurrent distribution. The contents of each tube were analyzed by paper chromatography (*n*-butanol/water). The results are given in Fig. 1. It may be seen that the distribution had partially separated the growth factors. Further distribution was carried out with the combinations marked I, II, III, and IV in Fig. 1. The results were those of Fig. 2. The fractions were then combined into three groups: A, B, and C (Fig. 2) and the components of each separated by chromatography on starch columns. From A and B, three fractions were obtained and from C only two. Each of these was analyzed by paper chromatography using isoamyl alcohol/phosphate buffer. The results are given in Table I.

^{*} Pyrocell Manufacturing Company, 207 East 84th Street, New York.

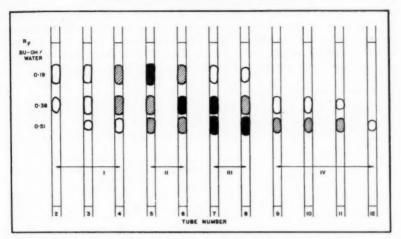


Fig. 1. Fractionation of growth factors by countercurrent distribution (isobutanol/acetic acid).

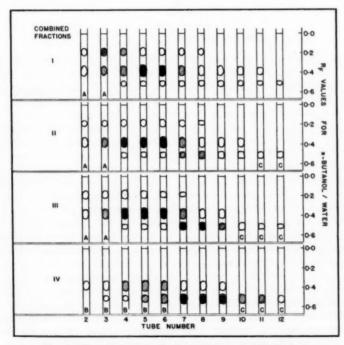


Fig. 2. Further fractionation by countercurrent distribution.

It is evident that the first component of fractions A and B was not homogeneous but contained at least two compounds; one single growth factor occurred in each of the others. The first component was separated into two parts by chromatography on paper sheets with n-butanol/ammonia. Each new part contained one factor with R_F 0.05 and 0.13 respectively.

TABLE I
SEPARATION OF COMPONENTS OF FRACTIONS A, B, AND C ON STARCH COLUMNS AND ANALYSIS OF THESE COMPONENTS BY PAPER CHROMATOGRAPHY

	R_F					
Fraction	Starch column with n-butanol/water	Paper strips with isoamyl alcohol/ phosphate buffer				
A	0.20* 0.38 0.51	0.58 to 0.66 0.75 0.71				
В	0.20 0.38* 0.51	0.59 to 0.66 0.74 0.71				
С	0.38	0.74 0.71				

^{*} Most abundant component.

Thus, four solutions had been obtained: I from fraction C (R_F n-butanol/ water 0.51), II from fraction B (R_F 0.38), III from fraction A (R_F nbutanol/ammonia 0.13), and IV from fraction A (R_F n-butanol/ammonia 0.05). The ultraviolet absorption spectra of these solutions were found to have the general appearance of spectra of derivatives of purines and pyrimidines. R_F values for the factors of the four solutions were determined with four different solvent systems. A number of purine and pyrimidine derivatives was tested for their ability to replace vitamin B12 as a growth factor for Lactobacillus leichmannii; only desoxyribosenucleosides showed strongly positive tests (see Table II). The desoxyribosenucleotides showed very slight ability to replace vitamin B₁₂. Two explanations are possible: (1) the nucleotides are weak growth factors themselves, or (2) these compounds become active only after conversion into the corresponding nucleosides. The latter was borne out by the change in activity of solutions of nucleotides upon standing: thymidylic acid in aqueous solution, stored at 2° C. for three weeks, increased its activity from practically zero to that of thymidine. It was demonstrated by paper chromatography that the desoxyribosenucleotide had been converted into the nucleoside.

The desoxyribosenucleosides were also chromatographed with the four solvent systems, and finally, mixtures of the fractions with the compounds they were thought to contain were chromatographed. From the results given in Table III, it was concluded that fractions I and II contained the

TABLE II

Purine and pyrimidine derivatives tested for ability to replace vitamin B_{12} for Lactobacillus leichmannii*

Purine or	Growth test carried out with							
pyrimidine	Free base	Desoxyribose- nucleoside	Desoxyribose- nucleotide	Ribose- nucleoside	Ribose- nucleotide			
Adenine	_	+	±	_	_			
Guanine	_	+	±	-	-			
Hypoxanthine	_	+	**	_	_			
Xanthine	_	**	**	_	_			
Cytosine	_	+	+	_	_			
5-Methyl-cytosine	_	+	±	**	**			
Uracil	_	+	**	_	_			
Thymine	_	+	+	**	**			
6-Methyl-uracil	_	**	**	**	**			

* Medium: Disco-CS vitamin B₁₂ agar.

** Compound not available.

+ Positive growth test.

+ Very slightly positive test.

- Negative growth test.

TABLE III

 R_F values of fractions and pure desoxyribosenucleosides

	Solvent system							
Compound	BuOH/water	BuOH/ammonia	Isoamyl alcohol /Na ₂ HPO ₄	BuOH/ethyl acetate/morpho- line				
Desoxyriboside of:								
Guanine	0.21	0.15	0.58	0.54				
Hypoxanthine	0.23	0.12	0.66	0.50				
Cytosine	0.23	0.28	0.73	0.67				
5-Methyl-cytosine	0.25	0.32	0.80	0.70				
Adenine	0.43	0.45	0.42	0.77				
Uracil	0.41	0.25	0.75	0.91				
Thymine	0.51	0.48	0.71	0.95				
Xanthine		0.05*	_	-				
Fraction								
1	0.51	0.50	0.71	0.95				
11	0.39	0.28	0.75	0.89				
111	0.22	0.10	0.66	0.52				
IV	0.20	0.05	0.60	_				
Fraction								
I+TDR	0.51	0.48	0.71	0.96				
II+UDR	0.39	0.28	0.75	0.88				
III+HDR	0.23	0.13	0.67	0.52				

* From McNutt, W. S. Biochem. J. 50: 384. 1952.

desoxyribosides of thymine and uracil respectively. Chromatography of fraction III always produced elongated zones, indicating lack of homogeneity. With n-butanol/ammonia, these zones could be divided into two, not clearly separate, fractions with R_F 0.12 and 0.18. The first component could not be distinguished from the desoxyriboside of hypoxanthine and the second showed the same chromatographic behavior as guanine desoxyriboside. The component of fraction IV is probably identical with xanthine desoxyriboside, inasmuch as its R_F value for n-butanol/ammonia agreed with the value given in the literature (8) for that compound and no other nucleoside with the same low R_F value was known. Definite proof could not be obtained because the pure compound was not available.

Discussion

When the results of this investigation are compared with data on beef liver (6, 7, 9, 13, 14) it may be seen that the total vitamin B_{12} activity of both materials is due mainly to the presence of cyano- and hydroxo-cobalamin and for a small part to additional growth factors. The vitamin B₁₂ activity of dried cod-liver residue is about as high as that of dried beef liver.

The additional growth factors are desoxyribosenucleosides in cod liver, as well as in beef liver, but whereas for materials of mammalian origin the derivatives of adenine, guanine, and cytosine are expected (1, 15), the codliver residue was found to contain the desoxyribosides of hypoxanthine, uracil, and xanthine. It has not been established whether these compounds were present in vivo or were artifacts, formed by deamination and oxidation. The latter seems probable since the derivatives of adenine, guanine, and cytosine were found in preliminary work with fresh cod livers (5).

It may be concluded that the vitamin B₁₂ activity and its distribution are very similar for cod-liver residue and beef liver, hence that cod-liver residues may be used to replace beef liver as a source of vitamin B12 in medicinal preparations and stock feeds.

References

- 1. CHARGAFF, E., VISCHER, E., DONIGER, R., GREEN, C., and MISANI, F. J. Biol. Chem.

- CHARGAFF, E., VISCHER, D.,
 177: 405. 1949.
 CRAIG, L. C. J. Biol. Chem. 155: 519. 1944.
 CRAIG, L. C. and Post, O. Anal. Chem. 21: 500. 1949.
 EDMAN, P. Acta Chem. Scand. 2: 595. 1948.
 GAGE, D. G. A study of the animal protein factor in cod-l University, Halifax, N.S. 1952.

 Vand Vandenheuvel, F. A. J. Fisheries A study of the animal protein factor in cod-liver residue. Thesis, Dalhousie
- 6. GUTTMANN, A. and VANDENHEUVEL, F. A. J. Fisheries Research Board Can. 9: 129. 1952
- HOFFMANN, C. E., STOKSTAD, E. L. R., HUTCHINGS, B. L., DORNBUSCH, A. C., and JUKES, T. H. J. Biol. Chem. 181: 635. 1949.
 MCNUTT, W. S. Biochem. J. 50: 384. 1952.
 PEELER, H. T., YACOWITZ, H., and NORRIS, L. C. Proc. Soc. Exptl. Biol. Med. 72: 515.

- Tennent, D. M., Whitla, J. B., and Florey, K. Anal. Chem. 23: 1748. 1951.
 Truscott, B., Gage, D. G., and Hoogland, P. L. J. Fisheries Research Board Can. 9: 129. 1952.
- 12. VANDENHEUVEL, F. A. 1952. U.S. Patent No. 2,588,338.
- Winsten, W. A. and Eigen, E. J. Biol. Chem. 181: 109. 1949.
 Woodruff, H. B. and Foster, J. C. J. Biol. Chem. 183: 569. 1950.
 Wyatt, G. R. Biochem. J. 48: 584. 1951.

THE ROLE OF A-ESTERASE IN THE ACUTE TOXICITY OF PARAOXON, TEPP, AND PARATHION1

By A. R. MAIN²

Abstract

The liver and serum A-esterase activity was determined in normal rats and in rats which four days previously had been fed a massive sublethal dose of aldrin. Aldrin pretreatment doubled the A-esterase activity in the liver and halved it in the serum. The intravenous toxicity of paraoxon increased slightly following aldrin pretreatment, while the oral toxicity decreased threefold. These results indicated that the A-esterase activity of the liver mediates the oral toxicity, and that the serum activity influences the intravenous toxicity. The latter conclusion was verified by the preparation of a partially purified concentrate of rabbit serum A-esterase. The concentrate was intravenously injected into rats. The serum A-esterase activity increased three- to five-fold, and the acute toxicity of paraoxon decreased significantly. Following aldrin pretreatment the oral toxicity of TEPP decreased significantly, while the intravenous toxicity remained unaltered. This result is explained by the relative activity of A-esterase toward paraoxon and TEPP. Parathion intravenous toxicity increased two- to three-fold following aldrin pretreatment. Comparison of paraoxon with parathion experiments suggested that aldrin pretreatment results in the inhibition of the 'in vivo' conversion of parathion to paraoxon. The serum esterase hydrolyzing phenylbenzoate in rat and rabbit was found to be the B-type esterase of Aldridge.

Introduction

The role of the paraoxon-hydrolyzing A-esterase of Aldridge (2) in the acute toxicity of paraoxon*, parathion†, and TEPP‡ was examined. Mounter (11) has reported that A-esterase hydrolyzes TEPP at approximately one third the rate of paraoxon hydrolysis, and that it does not hydrolyze parathion. Diggle and Gage (5) have shown that parathion does not inhibit cholinesterase, but Gage (7) has reported the 'in vivo' conversion of parathion in rat liver to the potent cholinesterase inhibitor, paraoxon. Diggle and Gage (6) have further reported that Dr. M. W. Goldblatt and Mr. J. Frodsham have indicated that removal of the liver does not make the rat less sensitive to parathion. Ball et al. (3) have observed that the oral LD₅₀ of parathion increased six to eight times in rats which four days previously had been fed a massive sublethal dose of aldrin. In the present work the serum and liver A-esterase activity of normal and aldrin pretreated rats was determined, together with the acute oral and intravenous toxicities of parathion, paraoxon, and TEPP. The variation in the A-esterase activity following aldrin pretreatment was related to the modifications in the acute oral and intravenous toxicities of these organophosphates. As a result the role of A-esterase in the toxicity of these

¹Manuscript received November 17, 1955. Contribution of the Department of National Health and Welfare, Occupational Health Division, 200 Kent St., Ottawa, Ontario.

³Present address Sir William Dunn Institute of Biochemistry, University of Cambridge, Cambridge, England.

^{*}Paraoxon—dielhyl-p-nitrophenyl phosphate. †Parathion—0,0-diethyl-0-p-nitrophenyl thiophosphate.

TTEPP—tetraethyl pyrophosphate. \$Aldrin—1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4,5,8-dimethanonaphthalene.

compounds has been elaborated. In contradiction to the conclusion of Ball et al. (3) the present work suggests that aldrin pretreatment acts to inhibit the conversion of parathion to paraoxon. The results indicate that the lethal conversion of parathion to paraoxon occurs at sites other than the liver.

A partially purified concentrate of rabbit serum A-esterase was prepared. This preparation was injected intravenously into rats and its effect on the intravenous paraoxon toxicity was measured.

The increase in the oral LD₆₀ of parathion in aldrin pretreated rats was associated by Ball *et al.* (3) with the slight rise in serum esterase activity which Crevier *et al.* (4) had observed. The method of Gomori (8) employing a phenylbenzoate substrate at pH 6.3 had been used. Neither Ball nor Crevier reported the identity of the esterase which Crevier *et al.* had determined. If the rise measured was due to A-esterase activity, it could be logically related to the increased LD₅₀ of parathion. Consequently in the present work the nature of the rat serum esterase hydrolyzing phenylbenzoate according to the method of Gomori was examined.

Rider, Moeller, and Dubois (14) have shown that in human serum most of the activity towards phenylbenzoate is due to cholinesterase. In the present work it is shown that this is not the case with rat serum. At pH 6.3, as specified by Gomori and as employed by Rider *et al.*, none of the activity in rat serum can be attributed to serum cholinesterase. By employing paraoxon inhibitor, it is shown that all the activity in rat serum at pH 6.3 is the B-type esterase of Aldridge. When rabbit serum, which is low in B-esterase and high in A-esterase activity, was examined, it was found that 95% of the activity toward phenylbenzoate was due to B-esterase. It was evident therefore that phenylbenzoate at pH 6.3 is very readily hydrolyzed by B-esterase but not by A-esterase.

The association between the rise in Gomori-esterase activity and the increased oral LD₅₀ which Ball *et al.* (3) implied does not therefore appear to have any logical justification. This conclusion is confirmed by the observation in the present work that aldrin pretreatment results in a decrease in the serum A-esterase activity.

The results of Rider, Moeller, and Dubois may be explained by the observation of Aldridge (1) that human serum contains no B-esterase. It is suggested that the Gomori method may be of use in determining B-esterase as well as serum cholinesterase. In rat and rabbit sera, and in rat liver, the method may be used to determine the B-esterase activity with a high degree of specificity.

Experimental

Materials

Sprague-Dawley male rats raised in a temperature controlled animal room were used. Diet was Master Fox Feed Breeder Starter Cubes.

Aldrin—a recrystallized product of 99% purity was used.

Paraoxon and parathion were both obtained from Albright and Wilson* in a purified form. The small amount of p-nitrophenol in the paraoxon was removed following the method of Aldridge (2) by shaking a 10% solution of paraoxon in chloroform with an aqueous solution of sodium bicarbonate.

TEPP was obtained from the Canada Defence Research Board in a waterclear purified form.

Naphthanil diazo red B salt was obtained from DuPont. The salt is diazotized 5-nitro-2-aminoanisole.

Phenylbenzoate—Eastman No. 632, phenol free phenylbenzoate was used. Eserine sulphate was a commercial product obtained from B.D.H.

Methods

Control and test groups, consisting of 10 rats each, were usually selected by random numbers. Occasionally selection was made by mixing the rats in a single cage and then distributing them one at a time in rotation to the various groups.

Aldrin Pretreatment

Aldrin was given orally to unstarved rats in corn oil (30 mgm. aldrin/ml.) at a level of 30 mgm./kgm. It was administered by hypodermic syringe. The needle, fitted with a pierced ball tip, was inserted into the stomach and the dose released. The same technique of administration was used for all oral dosing. On the fourth day after aldrin administration, various subsequent treatments were given.

Intravenous Injection

Intravenous injections were made into the saphenous vein, usually of the left leg. A 0.25 cc. hypodermic syringe calibrated to 0.01 cc. and fitted with a No. 25 hypodermic needle was used. The volume of poison injected varied from 0.08 to 0.25 cc. Injection time for each dose of poison was approximately 10 to 20 sec.

Measurement of A-esterase Activity Using Paraoxon Substrate

Both the colorimetric and manometric methods used to determine the activity in rat serum and liver have been described in detail by Aldridge (2). In the manometric determinations 0.50 ml. rat serum or 0.125 ml. rat liver (0.250 ml. liver homogenate) were used for each measurement. In the colorimetric determinations 0.20 ml. serum and 0.50 ml. liver were used.

Both tissue and non-enzymic hydrolysis blanks were run with the manometric determinations. The activity of the liver was always determined on the day of sacrifice since the activity in the liver of aldrin pretreated rats decreased rapidly with standing.

^{*} The author wishes to thank Messrs. Albright and Wilson for a generous supply of paraoxon and parathion.

A stock solution of paraoxon was prepared by dissolving 1.60 gm. paraoxon in methanol and diluting the solution to 10 ml. To make the buffered substrate, 1 ml. of this stock solution was blown into 50 ml. of either the bicarbonate or phosphate buffers.

The blood from each rat was taken by heart puncture in a heparinized syringe and centrifuged. The serum from each group was pooled, each rat contributing 1 ml. of serum. After bleeding the rat was stunned and the liver removed. The livers were immediately chilled in a beaker surrounded by ice. The livers of each group were weighed, and then minced together in a Waring Blendor for two minutes with an equal weight of distilled water.

The liver homogenate and the pooled serum, after suitable dilution with 1% w/v gelatin solution, were used for the measurement of A-esterase activity.

Determination of Esterase Activity Using Phenylbenzoate Substrate

The method of Gomori was modified for the determination of rat and rabbit serum and rat liver activity. Conveniently measured amounts of phenol were liberated by 2 λ of rat serum, 20 λ of rabbit serum, and 0.1 λ of liver (0.2 λ of homogenate). The serum or liver was diluted in distilled water and 1 ml. of the dilution was used for each determination.

M/15 phosphate buffer (pH 6.3) was prepared by dissolving 4.00 gm. $\rm Na_2HPO_4.7H_2O$ and 7.00 gm. $\rm NaH_2PO_4.H_2O$ in water and diluting to 1 liter. One milliliter of phenylbenzoate stock solution (0.800 gm. phenylbenzoate in 100 ml. methanol) was blown into 250 ml. of the buffer at 24 \pm 2°C. The buffered substrate was prepared daily, just before use. On standing or cooling, phenylbenzoate crystallized out of solution irreversibly. The activity was a function of the phenylbenzoate concentration and the effective concentration varied slightly from one batch of substrate to the next. These were related by an arbitrarily selected esterase standard which consisted of 1 ml. rat serum diluted to 500 ml. in water.

Diazo dye was prepared by dissolving 0.300 gm. naphthanil diazo red B salt in cold water, adding 2 ml. of 2% sulphuric acid, and diluting to 100 ml. The dye solution was kept cold.

Five milliliters of buffered substrate was pipetted into each of two 10-ml. volumetric flasks, A and B. The flasks were placed in a 37° C. water bath. After the substrate had reached temperature equilibrium, 1 ml. of enzyme dilution was added to flask A, and the time noted. Thirty minutes later 3.7 ml. of borax solution (saturated borax in 15% ethanol) was added to flask A. The flask was chilled. Flask B was then removed, and 1 ml. of enzyme dilution added, followed immediately by 3.7 ml. of borax solution. Diazo dye (0.4 ml.) was added to both flasks. The flasks were made to volume with water and shaken. The color was read at 525 m μ in a Fisher Electrophotometer. The value obtained for flask B was subtracted from the reading for flask A to correct for non-enzymic hydrolysis. When a 20 λ pipette was used to make the serum dilution, the method had a precision of \pm 2.0%, as judged from duplicate determinations using the serum from 10 different rats.

Eserine Inhibition

A solution of eserine 12 times the final concentration desired was prepared in phosphate buffer. An equal volume of eserine solution and serum dilution of twice the normal concentration was mixed and incubated 30 min. at 37° C. One milliliter of the incubated solution was then added to a digest flask, and the procedure described above was followed.

Paraoxon Inhibition

The procedure was the same as used for eserine inhibition.

Results

Effect of Aldrin Pretreatment on A-esterase Activity of Serum and Liver

The manometric and colorimetric methods of Aldridge (2) were used to determine the activity.

On the day of aldrin dosing the average weight of the rats in the aldrin and control groups was within a few grams of each other, but after dosing the aldrin groups either lost weight, or did not gain as fast as the controls. By the fourth day the aldrin group weighed 10 to 20% less than the control group. This body loss was not accompanied by a loss in liver weight, as shown in Table I.

Table I shows the typical reaction of the serum and liver A-esterase activity to aldrin pretreatment. The liver A-esterase activity increased approximately twofold, while the serum activity fell to one-half the control.

TABLE I

Effect of aldrin pretreatment on the A-esterase activity of rat serum and liver

(Each group consisted of 10 rats)

Group	Av. ra	at v	vt. ±	S.D.	,* 1	gm.	Av. liver wt., day	A	A-esteras		ctivity 30 min	, μ	l.
Cioup	Day of adr			Da			of sacrifice	Se	rur			ive	r
Control	256	±	6	280	±	13	12.6	453	±	15‡	1390	±	60‡
Aldrin	255	±	5	264	±	15	12.4	221	±	18‡	2620	±	24‡
Control	-	_		261	±	16	_	402	±	6†		_	
Aldrin	-	_		233	±	20	_	207	±	10†		_	
								Mgm.	p-n	itrophe	nol/ml	./3	0 mir
Control	240	±	7	246	±	11	10.4	2.70) :	t Ot	1	8.5	8
Aldrin	235	±	8	228	±	15	11.8	1.6	3 :	£ 0.08	1	7.6	

^{*} S.D.—standard deviation.

[†] Average of two determinations. Average of three determinations.

compounds has been elaborated. In contradiction to the conclusion of Ball et al. (3) the present work suggests that aldrin pretreatment acts to inhibit the conversion of parathion to paraoxon. The results indicate that the lethal conversion of parathion to paraoxon occurs at sites other than the liver.

]

a

ren

par

cle

dia

Me

by

va

at

Th

the

ora

qu

lef

a

fre

10

ac

In

(0

m

m

de

A partially purified concentrate of rabbit serum A-esterase was prepared. This preparation was injected intravenously into rats and its effect on the intravenous paraoxon toxicity was measured.

The increase in the oral LD_{50} of parathion in aldrin pretreated rats was associated by Ball *et al.* (3) with the slight rise in serum esterase activity which Crevier *et al.* (4) had observed. The method of Gomori (8) employing a phenylbenzoate substrate at pH 6.3 had been used. Neither Ball nor Crevier reported the identity of the esterase which Crevier *et al.* had determined. If the rise measured was due to A-esterase activity, it could be logically related to the increased LD_{50} of parathion. Consequently in the present work the nature of the rat serum esterase hydrolyzing phenylbenzoate according to the method of Gomori was examined.

Rider, Moeller, and Dubois (14) have shown that in human serum most of the activity towards phenylbenzoate is due to cholinesterase. In the present work it is shown that this is not the case with rat serum. At pH 6.3, as specified by Gomori and as employed by Rider *et al.*, none of the activity in rat serum can be attributed to serum cholinesterase. By employing paraoxon inhibitor, it is shown that all the activity in rat serum at pH 6.3 is the B-type esterase of Aldridge. When rabbit serum, which is low in B-esterase and high in A-esterase activity, was examined, it was found that 95% of the activity toward phenylbenzoate was due to B-esterase. It was evident therefore that phenylbenzoate at pH 6.3 is very readily hydrolyzed by B-esterase but not by A-esterase.

The association between the rise in Gomori-esterase activity and the increased oral LD₅₀ which Ball *et al.* (3) implied does not therefore appear to have any logical justification. This conclusion is confirmed by the observation in the present work that aldrin pretreatment results in a decrease in the serum A-esterase activity.

The results of Rider, Moeller, and Dubois may be explained by the observation of Aldridge (1) that human serum contains no B-esterase. It is suggested that the Gomori method may be of use in determining B-esterase as well as serum cholinesterase. In rat and rabbit sera, and in rat liver, the method may be used to determine the B-esterase activity with a high degree of specificity.

Experimental

Materials

Sprague-Dawley male rats raised in a temperature controlled animal room were used. Diet was Master Fox Feed Breeder Starter Cubes.

Aldrin—a recrystallized product of 99% purity was used.

Paraoxon and parathion were both obtained from Albright and Wilson* in a purified form. The small amount of p-nitrophenol in the paraoxon was removed following the method of Aldridge (2) by shaking a 10% solution of paraoxon in chloroform with an aqueous solution of sodium bicarbonate.

TEPP was obtained from the Canada Defence Research Board in a waterclear purified form.

Naphthanil diazo red B salt was obtained from DuPont. The salt is diazotized 5-nitro-2-aminoanisole.

Phenylbenzoate—Eastman No. 632, phenol free phenylbenzoate was used. Eserine sulphate was a commercial product obtained from B.D.H.

Methods

Control and test groups, consisting of 10 rats each, were usually selected by random numbers. Occasionally selection was made by mixing the rats in a single cage and then distributing them one at a time in rotation to the various groups.

Aldrin Pretreatment

Aldrin was given orally to unstarved rats in corn oil (30 mgm. aldrin/ml.) at a level of 30 mgm./kgm. It was administered by hypodermic syringe. The needle, fitted with a pierced ball tip, was inserted into the stomach and the dose released. The same technique of administration was used for all oral dosing. On the fourth day after aldrin administration, various subsequent treatments were given.

Intravenous Injection

Intravenous injections were made into the saphenous vein, usually of the left leg. A 0.25 cc. hypodermic syringe calibrated to 0.01 cc. and fitted with a No. 25 hypodermic needle was used. The volume of poison injected varied from 0.08 to 0.25 cc. Injection time for each dose of poison was approximately 10 to 20 sec.

Measurement of A-esterase Activity Using Paraoxon Substrate

Both the colorimetric and manometric methods used to determine the activity in rat serum and liver have been described in detail by Aldridge (2). In the manometric determinations 0.50 ml. rat serum or 0.125 ml. rat liver (0.250 ml. liver homogenate) were used for each measurement. In the colorimetric determinations 0.20 ml. serum and 0.50 ml. liver were used.

Both tissue and non-enzymic hydrolysis blanks were run with the manometric determinations. The activity of the liver was always determined on the day of sacrifice since the activity in the liver of aldrin pretreated rats decreased rapidly with standing.

^{*} The author wishes to thank Messrs. Albright and Wilson for a generous supply of paraoxon and parathion,

A stock solution of paraoxon was prepared by dissolving 1.60 gm. paraoxon in methanol and diluting the solution to 10 ml. To make the buffered substrate, 1 ml. of this stock solution was blown into 50 ml. of either the bicarbonate or phosphate buffers.

The blood from each rat was taken by heart puncture in a heparinized syringe and centrifuged. The serum from each group was pooled, each rat contributing 1 ml. of serum. After bleeding the rat was stunned and the liver removed. The livers were immediately chilled in a beaker surrounded by ice. The livers of each group were weighed, and then minced together in a Waring Blendor for two minutes with an equal weight of distilled water.

The liver homogenate and the pooled serum, after suitable dilution with 1% w/v gelatin solution, were used for the measurement of A-esterase activity.

Determination of Esterase Activity Using Phenylbenzoate Substrate

The method of Gomori was modified for the determination of rat and rabbit serum and rat liver activity. Conveniently measured amounts of phenol were liberated by 2 λ of rat serum, 20 λ of rabbit serum, and 0.1 λ of liver (0.2 λ of homogenate). The serum or liver was diluted in distilled water and 1 ml. of the dilution was used for each determination.

M/15 phosphate buffer (pH 6.3) was prepared by dissolving 4.00 gm. Na₂HPO₄.7H₂O and 7.00 gm. NaH₂PO₄.H₂O in water and diluting to 1 liter. One milliliter of phenylbenzoate stock solution (0.800 gm. phenylbenzoate in 100 ml. methanol) was blown into 250 ml. of the buffer at $24 \pm 2^{\circ}$ C. The buffered substrate was prepared daily, just before use. On standing or cooling, phenylbenzoate crystallized out of solution irreversibly. The activity was a function of the phenylbenzoate concentration and the effective concentration varied slightly from one batch of substrate to the next. These were related by an arbitrarily selected esterase standard which consisted of 1 ml. rat serum diluted to 500 ml. in water.

Diazo dye was prepared by dissolving 0.300 gm. naphthanil diazo red B salt in cold water, adding 2 ml. of 2% sulphuric acid, and diluting to 100 ml. The dye solution was kept cold.

Five milliliters of buffered substrate was pipetted into each of two 10-ml. volumetric flasks, A and B. The flasks were placed in a 37° C. water bath. After the substrate had reached temperature equilibrium, 1 ml. of enzyme dilution was added to flask A, and the time noted. Thirty minutes later 3.7 ml. of borax solution (saturated borax in 15% ethanol) was added to flask A. The flask was chilled. Flask B was then removed, and 1 ml. of enzyme dilution added, followed immediately by 3.7 ml. of borax solution. Diazo dye (0.4 ml.) was added to both flasks. The flasks were made to volume with water and shaken. The color was read at 525 m μ in a Fisher Electrophotometer. The value obtained for flask B was subtracted from the reading for flask A to correct for non-enzymic hydrolysis. When a 20 λ pipette was used to make the serum dilution, the method had a precision of \pm 2.0%, as judged from duplicate determinations using the serum from 10 different rats.

Eserine Inhibition

A solution of eserine 12 times the final concentration desired was prepared in phosphate buffer. An equal volume of eserine solution and serum dilution of twice the normal concentration was mixed and incubated 30 min. at 37° C. One milliliter of the incubated solution was then added to a digest flask, and the procedure described above was followed.

Paraoxon Inhibition

1

1

n

l.
i.
ie
er
co
of
in.
ie
oing
as

as

s.

The procedure was the same as used for eserine inhibition.

Results

Effect of Aldrin Pretreatment on A-esterase Activity of Serum and Liver

The manometric and colorimetric methods of Aldridge (2) were used to determine the activity.

On the day of aldrin dosing the average weight of the rats in the aldrin and control groups was within a few grams of each other, but after dosing the aldrin groups either lost weight, or did not gain as fast as the controls. By the fourth day the aldrin group weighed 10 to 20% less than the control group. This body loss was not accompanied by a loss in liver weight, as shown in Table I.

Table I shows the typical reaction of the serum and liver A-esterase activity to aldrin pretreatment. The liver A-esterase activity increased approximately twofold, while the serum activity fell to one-half the control.

TABLE I

Effect of aldrin pretreatment on the A-esterase activity of rat serum and liver

(Each group consisted of 10 rats)

Group	Av. rat wt.	S.D.,* gm.	Av. liver wt., day	A-esterase ac CO ₂ /ml./3	tivity, µl.
Gloup	Day of aldrin admin.	Day of sacrifice	of sacrifice	Serum	Liver
Control	256 ± 6	280 ± 13	12.6	453 ± 15‡	1390 ± 60‡
Aldrin	255 ± 5	264 ± 15	12.4	221 ± 18‡	2620 ± 24
Control	_	261 ± 16	_	402 ± 6†	_
Aldrin	_	233 ± 20	-	207 ± 10†	
				Mgm. p-nitropher	nol/ml./30 mi
Control	240 ± 7	246 ± 11	10.4	2.70 ± 0†	8.58
Aldrin	235 ± 8	228 ± 15	11.8	1.63 ± 0.08†	17.6

^{*} S.D.—standard deviation.

[†] Average of two determinations.

Average of three determinations.

Intravenous and Oral Toxicity of Paraoxon

Intravenous Toxicity

All rats were dosed according to their weights on the day of dosing. The concentration of the paraoxon solution was 0.5 mgm./ml. in 5% ethanol.

Aldrin pretreatment resulted in a slight decrease in the LD₅₀ of paraoxon, as shown in Table II. A similar decrease was also observed in the preliminary experiments. This decrease may be attributed to the drop in the serum A-esterase activity shown in Table I. Table II also shows that the time to death of aldrin pretreated rats was slightly shorter than the death time of control rats at comparable dosages.

The death time is a measure of the effect of the A-esterase activity. When aldrin pretreatment halved the A-esterase activity, the death time was shorter. However, the effect of the A-esterase activity also depends on the length of time it has to act. When higher doses of paraoxon reduce the death time, the effect of the A-esterase is proportionately lower. Thus, the slightly faster death time for aldrin pretreated rats, while being barely noticeable at doses of 0.25 mgm./kgm., when the death time is about 10 min., is too small to be discernible at a level of 0.30 mgm./kgm., when the death time is about six minutes.

TABLE II

Intravenous acute toxicity of paraoxon in control and aldrin pretreated rats

Dose, mgm./kgm.	No. rats	% mortality	Min. to death	Av. rat wt.
Control groups				
0.20	9	22	11.5 ± 0.5	242 ± 7
0.25	10	50	10 ± 3	247 ± 6
0.30	10	100	6 ± 1	240 ± 12
Aldrin pretreated grou	ips			
0.20	10	40	10 + 3	199 + 14
0.25	10	90	9 ± 3*	227 ± 17
0.30	10	100	6 ± 3	221 ± 18

LD to of control groups, 0.24 mgm. paraoxon/kgm.

LDw of aldrin pretreated groups, 0.21 mgm. paraoxon/kgm.

Oral Toxicity

Paraoxon was given in corn oil at a concentration of 5 mgm./ml. The rats were starved 24 hr. previous to dosing.

The oral LD_{50} of the aldrin pretreated group was about three times that of the control group, as shown in Table III. The time of death was about one and one-half times greater for the aldrin pretreated group than for the control group. Since paraoxon administered orally passes from the gastrointestinal system into the liver, the increased liver A-esterase activity due to aldrin pretreatment results in the increased LD_{50} of paraoxon.

^{*} One rat died three hours after injection; this figure not included in average death time.

MAIN: ACUTE TOXICITY

TABLE III

ORAL ACUTE TOXICITY OF PARAOXON IN CONTROL AND ALDRIN PRETREATED RATS

(All rats were starved 24 hr. previous to dosing with paraoxon)

Dose, mgm./kgm.	No. rats	% mortality	Min. to death	Av. rat wt.
Control groups				
3.0	10	50	24 ± 17	212 ± 8
5.0	10	90	14 ± 9	208 ± 9
6.0	10	90	16 ± 8	214 ± 9
7.0	10	90	12 ± 5	209 ± 9
Aldrin pretreated grou	ips			
7.0	10	10	39	186 ± 13
10.0	10	50	38 ± 8	190 ± 8
12.0	10	80	28 ± 14	184 ± 22
15.0	10	100	35 ± 15	188 ± 18
Preliminary control gr	roups			
2.5	10	20		194 ± 8
5.0	9	78		199 ± 8
7.5	10	100		202 ± 8

LD to of control groups, 3.5 mgm. paraoxon/kgm.

LD to of aldrin pretreated groups, 10 mgm. paraoxon/kgm.

TABLE IV

Intravenous acute toxicity of parathion in control and aldrin pretreated rats (Parathion administered in 50% ethanol at a concentration of 5 mgm./ml.)

Dose, mgm./kgm.	No. rats	% mortality	Min. to death	Av. rat wt.
Control groups				
3.0	10	0	-	215 ± 11
3.5	17	17.6	16 ± 2	220 ± 15
4.0	23	65	14 ± 7	225 ± 18
4.5	10	100	12 ± 3	227 ± 15
5.0	8	100	11 ± 3	212 ± 22
Aldrin pretreated grou	ıps			
8.5	10	0		213 ± 10
9.0	10	70	17 ± 5	210 ± 18
10.0	10	100	13 ± 4	212 ± 12

 LD_{50} of control groups, 3.8 mgm. parathion/kgm. LD_{50} of aldrin pretreated groups, 9.0 mgm. parathion/kgm.

Intravenous Toxicity of Parathion

Parathion was administered at a concentration of 5 mgm./ml. in 50% ethanol solution. The intravenous LD50 in the aldrin pretreated group increased two to two and one-half times, while the time to death increased by two to three minutes as shown in Table IV. According to Ball et al. (3) the oral LD₅₀ increased six to eight times in aldrin pretreated rats.

The question arises, does the increase in the liver A-esterase activity account for the increase in the LD_{50} of parathion by the intravenous route? The following considerations suggest that the increased liver A-esterase activity did not modify the intravenous toxicity of parathion.

The lethal effect of parathion results from its 'in vivo' conversion to paraoxon (5, 6, 7). A-esterase activity could, therefore, modify the toxicity of parathion by hydrolyzing the paraoxon resulting from conversion. To increase the LD₅₀ through such a mechanism would require an increased A-esterase activity. As shown in Table I, aldrin pretreatment did increase the liver A-esterase activity, but decreased the serum activity. Therefore, the increased LD50 of parathion could only be explained on the basis of A-esterase if the intravenous parathion toxicity was modified by the liver A-esterase. Now, the intravenous LD₅₀ of paraoxon has been shown to be independent of the liver A-esterase activity. It is suggested that this condition holds because significant amounts of paraoxon can not be transported to the liver by the blood stream before death occurs. Death occurred at 10 ± 3 min. in both aldrin and control rats following an intravenous LD₅₀ dose of paraoxon. That is, in 10 \pm 3 min. sufficient amounts of paraoxon had not been transported to the liver to significantly decrease the toxicity. With intravenous parathion injection, death occurred in 13 \pm 4 min. in aldrin pretreated rats, and in 12 \pm 3 min. in control rats. The dosing levels were 10 mgm./kgm. and 4.5 mgm./kgm. respectively. Both levels were 10% greater than the LD50 and resulted in 100% mortality. Since the time to death by intravenous parathion injection in both aldrin pretreated and control rats was in the same range as the death time of paraoxon injected rats, it is concluded that the liver A-esterase activity did not modify the intravenous parathion toxicity.

The possibility that aldrin pretreatment increased the A-esterase activity at sites other than the liver, and thus modified the intravenous LD $_{\delta 0}$ of parathion, is improbable, since the intravenous LD $_{\epsilon 0}$ of paraoxon did not increase

with aldrin pretreatment.

The present work suggests that aldrin pretreatment inhibits the conversion of parathion to paraoxon. Other possibilities have been examined but found improbable. The various factors associated with assimilation were eliminated, since the increase in the LD_{50} of parathion was observed by the intravenous route. Variation in the A-esterase activity in the liver and elsewhere has also been eliminated by comparison with paraoxon. The characteristics of the toxicity modification, where the death time appears to be almost constant while the concentrations must be increased to obtain the same mortality, strongly suggest inhibition of the conversion reaction.

Oral and Intravenous Toxicity of TEPP

Oral Toxicity

The effect of aldrin pretreatment on the oral LD₅₀ of TEPP was re-examined using starved male rats. The results are given in Table V. Aldrin pretreatment resulted in an increased oral LD₅₀, which was not more than 1/10th that observed by Ball *et al.* (3). Ball *et al.* also reported that the modification

TABLE V

ORAL ACUTE TOXICITY OF TEPP IN CONTROL AND ALDRIN PRETREATED RATS

(All rats starved 24 hr. previous to TEPP dosing)

				Av. rat wt.		
Dose, mgm./kgm.	No. rats	% mortality	Min. to death	Day of TEPP dosing	Day of aldring	
Control groups						
2.0	10	60	>60*	204 ± 15	210 + 15	
2.5	10	70	37 + 7+	199 ± 9	204 + 10	
3.0	10	90	40 ± 13	201 ± 10	202 + 10	
4.0	10	100	28 ± 14	204 ± 14	209 ± 16	
Aldrin pretreated gro	oups					
2.0	10	0	****	158 + 20	206 + 12	
2.5	10	50	36 ± 121	171 + 23	207 + 15	
3.0	9	44.5	45 ± 20	163 ± 8	201 + 11	
4.0	10	90	53 + 22	163 + 17	206 + 1.	

LD to of control groups, 1.9 mgm, TEPP/kgm.

LDso of aldrin pretreated groups, 3.0 mgm. TEPP/kgm.

* Six died overnight.

† Average death time of four rats. Three died overnight. † Average death time of two rats. Three died overnight.

of the oral LD50 of parathion which resulted from aldrin pretreatment did not vary significantly between male and female rats. Therefore, it is assumed that the differences between the observations of Ball and those reported in Table V are not due to sex. It is suggested that the differences may be attributed to the use of starved rats in the present work and of unstarved rats in the experiments of Ball et al. Preliminary work with unstarved rats gave extremely erratic results with oral doses of TEPP. The slight increase in the oral LD50 of TEPP is attributed to the increase in the liver A-esterase activity following aldrin administration. The increase in the LD50 of TEPP was less than that observed for paraoxon. This is probably due to the slower rate of hydrolysis of TEPP by A-esterase, which is only one third the rate of hydrolysis of paraoxon (11).

Ball et al. considered that the modification of the parathion toxicity which they observed could not be brought about by the slowing down of the conversion rate. This conclusion was based on the observation that aldrin pretreatment resulted in a fivefold increase in the oral LD50 of TEPP in unstarved female rats. In view of the present work, the increase in the oral LD₅₀ of TEPP does not appear to justify this conclusion.

Intravenous Toxicity

The conclusions drawn from the above observations suggest that the aldrin pretreatment should not increase the intravenous LD50 of TEPP, and that any decrease should be less than that observed with paraoxon. Since the decrease observed with paraoxon was slight, it would be expected that the

TABLE VI

INTRAVENOUS ACUTE TOXICITY OF TEPP IN CONTROL AND ALDRIN PRETREATED RATS
(TEPP administered in a 40% propylene glycol solution at a concentration of 0.40 mgm./ml.)

Dose, mgm./kgm.	No. rats	% mortality	Min. to death	Av. rat wt.
Control groups				
0.25	4	0	Marine.	237 + 10
0.30	11	27	7 + 1	232 ± 18
0.35	5	100	$ \begin{array}{c} 7 \pm 1 \\ 5.5 \pm 1.5 \end{array} $	229 ± 20
Aldrin pretreated grou	ips			
0.25	4	0		20.3 ± 1.3
0.30	10 5	40	7 + 2.5	202 ± 14
0.35	5	100	$ \begin{array}{c} 7 \pm 2.5 \\ 5.4 \pm 2.8 \end{array} $	202 ± 23
Preliminary control gr	roups			
0.25	10	0	seems.	217 ± 20
0.30	11	63.5	7 + 3	227 + 27
0.35	11 5	100	$ 7 \pm 3 \\ 5 \pm 1.5 $	204 ± 15
0.40	1	100	3	191

LD₅₀ of control groups, 0.30 mgm. TEPP/kgm. LD₅₀ of aldrin pretreated groups, 0.30 mgm. TEPP/kgm,

aldrin pretreatment should have little or no effect on the intravenous LD $_{50}$ of TEPP. The observed effect of aldrin pretreatment on the intravenous LD $_{50}$ of TEPP is shown in Table VI. TEPP was given in a solution of 40% propylene glycol and 60% water at a concentration of 0.40 mgm./ml. Aldrin pretreatment had no significant effect on the intravenous LD $_{50}$ of TEPP.

Effect of Increased Serum A-esterase Activity on Paraoxon Toxicity

The conclusions previously reached depend on the assumption that A-esterase hydrolyzes paraoxon and TEPP 'in vivo' and that this hydrolysis significantly affects the toxicity of these poisons. Variation in the A-esterase activity and the concurrent modification in toxicity resulting from aldrin pretreatment tend to verify this assumption. To confirm further that 'in vivo' hydrolysis due to A-esterase activity may significantly affect the toxicity of these poisons, a partially purified concentrate of rabbit serum A-esterase was prepared. The concentrate was injected intravenously into rats to raise the serum A-esterase activity. The effect of this increased activity on the intravenous toxicity of paraoxon was determined, and the results compared with those obtained by aldrin pretreatment.

Preparation of a Partially Purified Concentrate of Λ-esterase from Rabbit Serum

Rabbit serum was the richest available source of A-esterase, having 10 to 20 times the activity of rat serum. Ammonium sulphate fractionation enriched the A-esterase activity eight to nine times with respect to protein. The final fraction was five to six times more active after dialysis. The concentrate injected was thus 50 to 100 times more active than rat serum.

The blood from male and female rabbits of varying pedigree and condition was used. The pooled blood from several freshly slaughtered animals was allowed to stand in the refrigerator about three days. The supernatant serum was then decanted and the remaining detritus removed by centrifuging at room temperature.

Determination of A-esterase Activity

A rapid colorimetric method suitable for the determination of A-esterase activity in solutions, such as rabbit serum and ammonium sulphate fractions, was developed. As in the colorimetric method of Aldridge (2), paraoxon was used as a substrate. However, the solvent extraction of the acidified digest employed by Aldridge was omitted, and digestion was terminated by the addition of 95% ethanol. No interfering cloudiness developed if the temperature was not permitted to fall below 25° C., and if the digest did not contain more than about five milligrams soluble protein. The color of the liberated *p*-nitrophenol was measured directly at the pH of the digest. The optical density was stable for one hour in the temperature range of 25° C. to 37° C. The method was rapid and easy, requiring about two to three minutes of manipulation for each determination.

Materials

All chemicals were Reagent Grade unless otherwise stated.

Phosphate buffer—16.00 gm. Na₂HPO₄.7H₂O and 1.20 gm. NaH₂PO₄.H₂O were dissolved and diluted to 1 liter with water. The pH was 7.6.

Gelatin—an aqueous 1% solution of Knox Brand Gelatin was used.

Paraoxon stock solution—1.250 gm. paraoxon, repurified according to the method of Aldridge (2), was diluted to 50 ml. with methanol.

Buffered substrate was prepared just before use. Two milliliters of 1% gelatin was diluted to 100 ml. with phosphate buffer in a 100 ml. volumetric flask. One milliliter paraoxon stock solution was blown into the gelatin-buffer mixture and thoroughly mixed by shaking. The final paraoxon concentration was $1.82 \times 10^{-3} M$. The buffered substrate was stable for about three days when stored at -5° C.

Procedure for the Determination of A-esterase Activity

Five milliliters of buffered substrate was pipetted into each of three or more 10-ml. volumetric flasks depending on the number of determinations to be made. One flask was used to zero the electrophotometer, one flask served to correct for non-enzymic hydrolysis and color from the enzymic sample, and the third flask was used for measuring the A-esterase activity. The second and third flasks were placed in a constant temperature water bath set at 37° C. and allowed to reach temperature equilibrium. One milliliter of the appropriate enzyme dilution was then added to the flask used for measuring the activity. Ten lambda of rabbit serum digested for 15 to 20 min. liberated a conveniently measured amount of *p*-nitrophenol. To terminate the digestion, the flask was removed from the water bath and made to volume

with 95% ethanol. After the same time interval the non-enzymic hydrolysis flask was removed from the bath. One milliliter of the enzyme dilution was added and the flask was immediately made to volume with ethanol. The optical density was measured with a Fisher Electrophotometer employing a 425 m μ filter at 'C' sensitivity. The cuvettes had a light path of 1 cm. Correction was made by subtracting the gammas of p-nitrophenol in the non-enzymic hydrolysis blank from that in the sample.

The rate of hydrolysis by 10λ of rabbit serum was linear to 30 min., but decreased thereafter. After 15 min. digestion, the *p*-nitrophenol liberated by rabbit serum was found to be linear over the range investigated, that is 0.5, 1, 2, 5, 10, 15, and 20λ .

Determination of Protein

Protein in native serum was determined by a micro-Kjeldahl method. Hydrogen peroxide was added to the sulphuric acid digestion medium to speed oxidation. The ammonia was steam distilled from the alkalinized digest, caught in boric acid, and titrated with sulphuric acid.

pi

pi

ti

tr

aş

pi

tl

pi

gi

T

3

Sa

CC

in

A

th

gi

a

in

to

in

Se

ha

Cá

fi

F

th

de

Protein in both native serum and in ammonium sulphate fractions was determined by the biuret method of Robinson and Hogden (14).

Procedure for the Preparation of a Partialty Purified Concentrate of A-esterase from Rabbit Serum

Seventy-five grams solid ammonium sulphate was stirred into 300 ml. of serum previously adjusted to a temperature of 15° C. Stirring was continued for 90 min. The mixture was centrifuged for one hour in an International Clinical Centrifuge (contained in a Frigidaire Deep Freeze) at about 0° C. The clear pink supernatant solution contained 85–90% of the initial A-esterase activity and about 70% of the protein. The precipitate was discarded. The supernatant (sp. gr. 1.160) was cooled to -5° C. in a 50 gal. Aminco Refrigerated Constant Temperature Bath, which became available during the final stages of the investigation. Twelve milliliters phosphoric acid mixture, prepared by mixing together 2 ml. sirupy phosphoric acid, 8 ml. 0.5 M NaH₂PO₄, 5 ml. saturated ammonium sulphate, and 5 ml. water, was then added by capillary with vigorous stirring. The pH dropped from 7.6 to 4.1. The mixture was centrifuged for one hour at approximately 0° C. The supernatant contained 40 to 50% of the protein and 5 to 10% of the A-esterase activity. The supernatant was discarded. The precipitate contained 80 to 85% of the A-esterase activity and 20 to 30% of the protein.

The precipitate was dissolved in cold 1% ammonium hydroxide and the volume was adjusted to about 70 ml. Sufficient cold saturated ammonium sulphate solution (30 ml.) was then added to make the specific gravity 1.160. The small amount of precipitate which formed was removed by centrifuging and discarded. The supernatant was cooled to -5° C. and sufficient phosphoric acid mixture was added to make the pH 4.8. The mixture was stirred for one hour and then centrifuged at about 0° C. for one hour or until the supernatant was clear. The precipitate contained 7 to 8% of the initial

TABLE VII

RESULTS FROM THE PROCEDURE FOR THE CONCENTRATION AND PARTIAL PURIFICATION OF RABBIT SERUM A-ESTERASE

Run	Final purifica	Enrichment		
No.	Serum	Before dialysis	After dialysis	factor*
40	0.067	0.55	0.45	8.3
41	0.071	0.67	0.55	9.3

^{*} Before dialysis.

protein and approximately 70% of the initial A-esterase activity. This procedure enriched the activity approximately ninefold with respect to the protein. The precipitate was taken up in 2 to 3 ml. of 0.5 M Na₂HPO₄ solution, the volume estimated in a 25 ml. graduate, and the mixture then transferred to a cellophane dialysis sock. The mixture was dialyzed overnight against running tap water, temperature 20–22° C. The small amount of protein left undissolved was removed by centrifuging. Sufficient sodium chloride was added to make the salt concentration 0.85%. After dialysis the activity was five to six times that of the starting serum. Dialyzing the precipitate resulted in a loss of 14 to 15% of the initial activity. Table VII gives a summary of the results.

The Effect of the Concentrate on the Rat Serum A-esterase Activity

Three male rats weighing 228 ± 3 gm. were used. Before the injection a 3 ml. sample of blood was removed by heart puncture. The serum of this sample was used to determine the initial activity of the rat serum. A-esterase concentrate was then injected into the saphenous vein. At varying time intervals 3 ml. samples of blood were again withdrawn by heart puncture. As many as three heart punctures were made within 45 min. without killing the rat. Indeed, the rats survived the experiment and subsequently continued growth.

Table VIII shows that within 5 to 15 min. of injection, the A-esterase activity of the serum increased two- to three-fold depending on the amount injected. If it is assumed that 5% of the body weight is serum, it is possible to calculate the theoretical maximum specific activity of the rat serum resulting from the injected A-esterase concentrate and the original activity of the serum. With the injection of 0.5 ml. of concentrate, the activity for a rat having an original activity of 15 microliters CO₂/ml. serum/minute was calculated as 40 microliters CO₂/ml./minute. As shown in Table VIII, this figure is in good agreement with that actually found 15 min. after injection. Forty-five minutes after injection, the A-esterase activity of the injected rats had dropped to 30–40% of the earlier (5–15 min.) activity. The injection of the A-esterase concentrate alone did not appear to have any permanent deleterious effects on the rats.

TABLE VIII

Intravenous injection of rabbit serum A-esterase concentrate into rats—the effect on the serum A-esterase activity at various times after injection

(Concentrate injected into the saphenous vein. Blood samples taken by heart puncture)

	Micro	MI.			
Rat No.	Before injection	5 Mi	n. after injecti 15	on 45	- A-esterase concentrate injected
1	300	624 660		441	0.3
2	450	*****	1350 1310	740	0.5
3	432	-	1305 1310	-	0.5

^{*} Activity of A-esterase concentrate injected, 16,500 microliter CO2/ml./30 min.

TABLE IX

h

Ŀ

a C w C d re sh

n d

a

as

th

ac

se

in

THE EFFECT OF PREINJECTED A-ESTERASE CONCENTRATE ON THE INTRAVENOUS TOXICITY OF PARAONON

A-esterase preparation used	Ml. A-esterase injected	Paraoxon dose, mgm./kgm.	No. of rats	c; mortality	Min. deat	
Control		0.25	10	50	9 +	3
Control	-	0.30	10	100	6 +	3
No. 41	1.0	0.30	7	4.3	11 ±	2
Control	***	0.25	4	50	10 ±	3
Control	_	0.30	3	100	5.5 ±	0.
No. 40	0.9	0.30	4	0	_	
Control	_	0.25	10	50	10 ±	3
Control	_	0.30	8	100	7 +	3
No. 39	0.8	0.30	2	0	_	
		wt. of injected rats		y of A-esterase NP* liberated		

		mg.m z
No. 41	213 ± 3	38.7
No. 40	201 ± 1	37.2
No. 39	187 ± 3	31.5

^{*} PNP-paranitrophenol.

Intravenous Injection of Paraoxon Following Injection of A-esterase Concentrate

The A-esterase concentrate was injected into the saphenous vein of the left leg, and two minutes later, paraoxon was injected into the saphenous vein of the right leg. As shown in Table IX, the mortality resulting from an intravenous paraoxon dose of 0.30 mgm./kgm. was reduced from 100 to 23% following injection of the concentrate. In those instances where A-esterase

injection followed by paraoxon resulted in death, the death time was increased about 180%. The results given in Table VIII suggest that the injected concentrate increased the rat serum A-esterase activity four to five times. It is concluded that increasing the serum A-esterase activity does decrease the intravenous toxicity of paraoxon. This decrease is considered to be in agreement with the results obtained by aldrin pretreatment for the following reasons.

An intravenous paraoxon dose of 0.30 mgm./kgm. killed control rats in an average of six minutes, with some rats dying in three minutes. The criterion of death was the last heart beat. However, one or two minutes before the heart stopped, the rat always went limp after which there was no recovery. For the purpose of considering the action of the A-esterase, the death time should perhaps be reduced by approximately one minute. In cases where rats went into convulsions before all the paraoxon had been injected, increasing the A-esterase activity could only prolong the death time. Thus, the mortality modification, while apparently small when compared to the four- to five-fold increase in A-esterase activity, is to be expected when the time factor is Moreover, the normal serum A-esterase activity is quite low. A four- or five-fold increase may not be very significant in terms of the rate at which paraoxon must be hydrolyzed to increase effectively the intravenous LD₅₀ of paraoxon. For example, when the serum A-esterase activity was halved by aldrin pretreatment, a barely detectable increase in paraoxon toxicity resulted.

Esterases Hydrolyzing Phenylbenzoate in Rat Serum

te

1e

us

ın

se

The increase in the oral LD₅₀ of parathion in aldrin pretreated rats was associated by Ball *et al.* (3) with the rise in serum esterase activity which Crevier *et al.* (4) measured. Crevier *et al.* used the method of Gomori (8) which employed a phenylbenzoate substrate at pH 6.3. Neither Ball nor Crevier reported the identity of the esterase in rat serum which they had determined. If the rise measured was due to A-esterase, it could be logically related to the increased LD₅₀ of parathion. However, it has already been shown that aldrin pretreatment decreases, rather than increases the serum A-esterase activity. It seems evident that the rise which Crevier *et al.* measured was not due to A-esterase. The association implied by Ball *et al.* does not therefore appear to have any logical justification. However, it was of interest to identify the esterase in rat serum responsible for this rise in activity.

To determine the contribution of serum cholinesterase, escrine was used as inhibitor. At $10^{-6}\,M$ escrine concentrations no significant inhibition resulted, as shown in Table X. Myers and Mendel (12) and many others have shown that $10^{-6}\,M$ escrine completely inhibits serum cholinesterases. None of the activity toward phenylbenzoate at pH 6.3 can therefore be attributed to serum cholinesterase.

At concentrations of eserine which were 10⁻³ M, 29% of the activity was inhibited at pH 6.3. It is suggested that under the conditions of the Gomori

TABLE X

EFFECT OF PARAOXON AND ESERINE ON THE HYDROLYSIS OF PHENYLBENZOATE AT pH 6.3 BY RAT SERUM

(No gelatin-pooled serum of 10 rats)

Inhibitor	Final concentration of inhibitor, M	
	1 × 10-7	2
Eserine	1×10^{-6}	0
	1×10^{-5}	29
	1×10^{-4}	98
Paraoxon	1×10^{-6} 1×10^{-5}	100
	1 × 10-5	100

method, this concentration of eserine inhibits aliesterase but not cholinesterase. Using p-nitrophenol esters, Huggins and Lappides (10) have shown that aliesterase inhibition by eserine is dependent on the amount of serum used in the determination of activity. They reported that 10-5 M eserine resulted in no inhibition when 0.10 ml. of rabbit serum was used, and in 60% inhibition when 0.01 ml. was employed. The Gomori method, as modified for the determination of esterase activity in the rat, employs 0.002 ml. of serum. It was concluded that the activity inhibited by 10⁻⁵ M eserine was aliesterase. In the present investigation the pI₅₀ of eserine was 4.9 when 0.02 ml. of rabbit serum was employed with phenylbenzoate substrate at pH 8.0. This is in accordance with the observation of Huggins and Lappides.

hy

inl

rai

Ale

ese

ch

be

thi

(1)

95

Ta ap

tha

be

the hu ph Ale hy

and res 25

ma res

The contribution of rat serum cholinesterase to the hydrolysis of phenylbenzoate at pH 8.0 was also examined. At this pH about 15% of the activity may be attributed to cholinesterase, as shown in Table XI.

TABLE XI

EFFECT OF ESERINE INHIBITION ON THE HYDROLYSIS OF PHENYLBENZOATE AT pH 8.0 (No gelatin-pooled serum of 10 rats)

Eserine concentration	Activity, mgm./ml./30 min.	% of contro
_	4.95	100
	4.95	100
5×10^{-8}	4.08	8.3
1×10^{-7}	4.29	87
5×10^{-7}	3.94	80
1×10^{-6}	4.50	91
2.5×10^{-6}	4.10	8.3
5 × 10-6	4.10	8.3
7.5 × 10-6	3.88	78
1 × 10-5	2.74	59
5 × 10-5	0.60	12
1×10^{-4}	0.16	6

TABLE XII

Hydrolysis of phenylbenzoate by rat and rabbit serum—the effect of $10^{-6}\ M$ paraoxon on the rate of hydrolysis

(Each value the average of two determinations)

Serum	рН	Conc. paraoxon	Activity, mgm. phenol/ml./30 min.	% inhibition
Rat	6.3	_	4.11	0
Rat	6.3	1×10^{-6}	0	100
Rabbit	6.3	_	0.46	0
Rabbit	6.3	1×10^{-6}	0.023	95
Rat	8.0	_	6.18	0
Rat	8.0	1×10^{-6}	0	100
Rabbit	7.6	PERSONAL PROPERTY AND ADDRESS OF THE PERSONAL PR	0.79	0
Rabbit	7.6	1×10^{-6}	0.23	59

Paraoxon was used to determine the contribution of A-esterase to the hydrolysis of phenylbenzoate. A concentration of 10-6 M resulted in complete inhibition as shown in Table X. It is concluded that none of the activity in rat serum toward phenylbenzoate at pH 6.3 can be attributed to A-esterase. Aldridge (1) has termed the serum esterases which are not inhibited by eserine, but are inhibited by paraoxon, the B-type esterases. Since the serum cholinesterase and A-esterase do not contribute to the hydrolysis of phenylbenzoate at pH 6.3 it is concluded that B-esterase is entirely responsible for this hydrolysis in normal rats.

Since rat serum is low in A-esterase activity and high in B-esterase activity (1) this result may be expected. However, when rabbit serum was examined, 95% of the activity at pH 6.3 could be attributed to B-esterase, as shown in Table XII. Since rabbit serum is low in B-esterase and high in A-esterase it appears that B-type esterases hydrolyze phenylbenzoate much more readily than do the A-type esterases.

The observation that B-esterase is responsible for the hydrolysis of phenylbenzoate at pH 6.3 in rat and rabbit serum is in apparent contradiction to the findings of Rider, Moeller, and Dubois (13). They reported that in humans serum cholinesterase is responsible for most of the hydrolysis of phenylbenzoate at pH 6.3. Gomori (9) also reported similar findings. Since Aldridge (2) has reported that human serum contains no B-type esterase, the hydrolysis of phenylbenzoate by cholinesterase in humans and by B-esterase in rats may be explained by species differences.

The effect of aldrin pretreatment on the B-esterase activity of rat serum and liver was investigated using phenylbenzoate substrate at pH 6.3. The results are given in Table XIII. The B-esterase activity increased 10 to 25% in both the liver and serum.

The effect of aldrin pretreatment on serum cholinesterase was measured manometrically using benzoylcholine as substrate. Aldrin pretreatment resulted in an increase of approximately 20% in serum cholinesterase activity.

TABLE XIII

B-ESTERASE ACTIVITY AFTER ALDRIN PRETREATMENT IN RAT LIVER AND SERUM AS MEASURED BY THE GOMORI METHOD

(Each group consisted of 10 rats)

	B-esterase mgm. phenol	e activity, /ml./30 min.	% of c	ontrol
Group	Serum	Liver	Serum	Liver
Control	4.50 ± 0.3	168 ± 9	100	100
Aldrin	5.04 ± 0.18	195 ± 2	113	116
Control	4.62 ± 0	181 ± 2	100	100
Aldrin	5.82 ± 0.33	199 ± 5	126	110

The effect of aldrin pretreatment on rat serum was to increase by approximately 20% both the B-esterase, as measured by the Gomori method, and the serum cholinesterase, as measured with a benzoylcholine substrate. Crevier et al. used the Gomori method at pH 6.3 to measure the rise in serum esterase following aldrin pretreatment. It has been shown that in rat serum at pH 6.3 none of the activity measured by the Gomori method is cholinesterase. It is concluded that the rise in esterase activity measured by Crevier et al. was due to B-esterase.

The Effect of Gelatin and Ether on the Gomori Esterase Activity and the Effect of Gelatin on Eserine Inhibited Digests

Blood for determinations by the method of Gomori was usually taken from the rat's tail, but occasionally it was obtained by heart puncture. Before the heart puncture, the rat was anesthetized by ether inhalation. When the activity of the serum taken by the two methods was compared, the activity of the serum from the heart puncture was consistently lower than that from the tail. The effect of ether anesthesia on the Gomori esterase activity was therefore measured and the results are given in Table XIV. The effect of adding gelatin to the digest was also determined. In the absence of gelatin, ether anesthesia resulted in a drop of 40% in the esterase activity. In the presence of gelatin, ether anesthesia increased the activity 8 to 10%. The effect on the activity of raising the pH from 6.3 to 8.0 was 60% greater in the presence of 0.1% gelatin than in its absence.

Certain concentrations of eserine were observed to increase the serum Gomori esterase activity in the presence of 0.1% gelatin. Eserine concentrations from $2.5 \times 10^{-8}~M$ to $1 \times 10^{-6}~M$ increased the activity by as much as 19% at pH 8.0. In the absence of gelatin no increase was observed. At pH 6.3 the activity increased by a maximum of 27.5% when eserine concentrations of $5 \times 10^{-7}~M$ to $5 \times 10^{-6}~M$ were used. The serum activity toward triacetin at pH 7.4 also increased when the eserine concentration was $5 \times 10^{-7}~M$ to $1 \times 10^{-5}~M$. The activity of rabbit serum toward phenylbenzoate at pH 8.0 with an eserine concentration of $1 \times 10^{-9}~M$ to $1 \times 10^{-6}~M$

Co

No

Eth

Etl

of eve

thi and str of int

ab

ora Wil ora tov dec

est

A-

TABLE XIV

EFFECT OF ETHER ANESTHESIA AND GELATIN ON THE HYDROLYSIS OF PHENYLBENZOATE
BY RAT SERUM

(Pooled serum of 10 rats)

	Acti	vity	~ .	
	Mgm. phenol/ml.	pH 8.0 V 100 -	% of control	
pН	/30 min.	pH 6.3 × 100	At pH 6.3	At pH 8.0
Control, no ether	, no gelatin			
6.3	4.09	117	-	
8.0	4.80	117		_
Ether, no gelatin				
6.3	2.34	117	59	
8.0	2.82	117		59
No ether, gelatin	0.1%			
6.3	4.60	128	111	
8.0	5.89	128		111
Ether and gelati	n 0.1%			
6.3	5.00	131	122	
8.0	6.52	131		135

increased as much as 44% in the presence of 0.1% gelatin. The significance of this increased activity in the presence of gelatin is not known. However, the increase must be considered when evaluating hydrolysis by cholinesterase using eserine inhibition.

Discussion

f

١,

e

n

n

1-

d.

1-

y

15

M

According to Mounter (11) rabbit serum A-esterase hydrolyzes paraoxon about 2.8 times faster than TEPP, but does not hydrolyze parathion. The ratios between the intravenous and oral LD60 of paraoxon, TEPP, and parathion are 14.5, 6.3, and 1.5 respectively. The correlation between these ratios and the relative activity of A-esterase toward these organophosphates strongly suggests that A-esterase is the principal detoxification mechanism of the body against organophosphates. The difference between the oral and intravenous LD₁₀ can be explained by the relatively high A-esterase activity of the liver and by the slower ingestion rates by the oral route. That the liver A-esterase is responsible for the difference observed by the intravenous and oral routes is given support by the evidence following aldrin administration. When the liver A-esterase activity was doubled by aldrin pretreatment, the oral LD₈₀ of paraoxon and TEPP increased according to their relative activity toward A-esterase, while the intravenous LD remained unchanged, or decreased slightly. The evidence obtained by the use of a partially purified concentrate of rabbit serum A-esterase confirms the observation that Aesterase activity significantly modifies paraoxon toxicity 'in vivo'.

differences between the oral and intravenous LD50 of parathion suggest that the paraoxon which results from parathion conversion in the liver is largely destroyed by the liver A-esterase. The net effect of the liver on parathion toxicity may be considered to be a detoxifying one. The results of aldrin pretreatment tend to confirm this suggestion.

Evidence from aldrin pretreatment also suggests that parathion is converted in the liver and at other sites, and that the conversion at these sites is chiefly responsible for lethal cholinesterase inhibition. In addition it appears that aldrin pretreatment inhibits this conversion of parathion to paraoxon at sites other than the liver. Whether such inhibition occurs in the liver is not known. Preliminary 'in vitro' experiments to demonstrate

this point were inconclusive.

The effect of aldrin pretreatment on the activity of A- and B-esterases was markedly different. In the serum, the B-esterase activity increased 10 to 25%, while the A-esterase activity decreased about 50%. In the liver, the B-esterase activity increased 10 to 25%, while the A-esterase activity increased by approximately 200%. The normal liver B-esterase activity is 30 times that of the serum. In comparison, normal liver A-esterase activity is about three times that of the serum. The markedly different reaction of the liver and serum A- and B-esterases to aldrin pretreatment, together with differences in distribution, suggest that the two esterases may also have fundamentally different physiological origins and functions.

The observation that phenylbenzoate is hydrolyzed chiefly by B-esterase in rat and rabbit serum and by cholinesterase in human serum (13) may be explained by differences in the esterase patterns of various species.

Acknowledgments

The author takes pleasure in acknowledging the valuable technical assistance of Mr. E. A. Belanger and Mr. J. W. Sinclair. He also wishes to thank Dr. H. N. MacFarland for the encouragement and support which he gave throughout the investigation.

References

 ALDRIDGE, W. N. Biochem, J. 53: 110. 1953.
 ALDRIDGE, W. N. Biochem, J. 53: 117. 1953.
 BALL, W. L., SINCLAIR, J. W., CREVIER, M., and KAY, K. Can. J. Biochem. Physiol. 32: 440.

- 4. CREVIER, M., BALL, W. L., and KAY, K. Arch. Ind. Hyg. and Occupational Med. 7:

CREVIER, M., BALL, W. L., and KAY, K. Arch. Ind. Hyg. and Occupational Med. 7: 152. 1953.
 DIGGLE, W. M. and GAGE, J. C. Biochem. J. 49: 491. 1951.
 DIGGLE, W. M. and GAGE, J. C. Nature, 168: 998. 1951.
 GAGE, J. C. Biochem. J. 54: 426. 1953.
 GOMORI, G. J. Lab. Clin. Med. 34: 275. 1949.
 GOMORI, G. J. Lab. Clin. Med. 42: 445. 1953.
 HUGGINS, C. and LAPPIDES, J. Biol. Chem. 170: 467. 1947.
 MOUNTER, L. A. J. Biol. Chem. 209: 813. 1954.
 MYERS, D. K. and MENDEL, B. Proc. Soc. Exptl. Biol. Med. 71: 357. 1949.
 RIDER, J. A., MOELLER, H. C., and DUBOIS, K. R. Proc. Soc. Exptl. Biol. Med. 76: 427. 1951.

14. ROBINSON, H. W. and HOGDEN, C. G. J. Biol. Chem. 135: 707. 1940.

THE EFFECT OF CHLORPROMAZINE GIVEN WITH REINFUSION ON THE MORTALITY RATE FROM STANDARDIZED HEMORRHAGIC SHOCK IN THE RATI

By G. F. CARRUTHERS2 AND C. W. GOWDEY

Abstract

t

)

1

9

s

0

e 1

S

t

S

e

e

e

e

2:

7.

Anesthetized rats were subjected to a standardized hemorrhagic shock procedure; one group (20 animals) served as controls and another group (eight animals) was injected with chlorpromazine. The treated animals died very quickly: only one survived more than 12 hr. and none 48 hr.; the average survival time of 18 control fatalities was 14.0 hr., and two lived. Of the various cardiovascular and respiratory indices measured, only the post-reinfusion arterial pressure was different in the treated from that in the control group. between the control groups in this and in an earlier series (Downie and Stevenson (1955)) can probably be accounted for by differences in the temperature of the laboratory.

Introduction

The purpose of this study was to evaluate the effect of chlorpromazine hydrochloride (RP4560, Largactil) on the circulatory failure resulting from prolonged hemorrhagic hypotension. Jaulmes, Laborit, and Benitte (7) reported in 1952 that a combination of chlorpromazine and lowering of the "central" temperature to 28-30° in dogs, with the aid of ice bags, was useful "in the prevention and probably also in the therapy of hemorrhagic shock". In the same year Fournel (6) and a year later Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet (3) claimed that chlorpromazine (2 mgm./kgm. given intravenously immediately after the bleeding) protected dogs against the shock induced by the Wiggers' technique and led to a higher 72-hr. survival rate in the treated group. They also stated that the subcutaneous administration of 2.5-5 mgm./kgm. chlorpromazine to rats protected them against the traumatic shock induced by the Noble-Collip drum. The above authors believed that the protection afforded by chlorpromazine could be attributed to its sympatholytic action.

The exact mechanism of the protection by sympathetic blockade in shock is not fully understood; at least some of the protection in the controlledhypotensive-level type of hemorrhagic shock in dogs may be attributable to a greater blood volume (and arterial oxygen transport) at a given arterial pressure in the treated animals.

Because of the beneficial results in shock reported by the French investigators and because chlorpromazine is said (3) to possess the dual properties of adrenergic blockade and reduction of oxygen consumption, it was decided to test the efficacy of chlorpromazine in controlled hemorrhagic hypotension in rats.

Manuscript received September 19, 1955.
Contribution from the Departments of Physiology and Pharmacology, University of Western Ontario, London, Canada. This work was supported by Defence Research Board of Canada, grant number 9310-20, project 050-93-10-20, to J. A. F. Stevenson and C. W. Gowdey.

²During the tenure of a Lederle Medical Student Research Fellowship.

Method

The method used was that devised by Downie and Stevenson (4). Adult rats of the Sprague-Dawley strain, weighing between 360 and 465 gm., were anesthetized with sodium pentobarbital (40 mgm./kgm.) injected intraperitoneally. The right jugular vein was cannulated and connected to a small reservoir and levelling bottle. The arterial pressure could thus be controlled by adjusting the height of the levelling bottle. The left carotid artery was cannulated and connected to a Sanborn electromanometer and "Polyviso" recorder. This system produced a continuous tracing from which the arterial blood pressure and heart rate could be read. Forty-five minutes after the onset of anesthesia a volume of blood sufficient to lower the rat's arterial pressure to 30 mm. Hg was withdrawn over a 10-min. period into the reservoir. This initial bleeding volume was recorded. As vasoconstriction occurred, further small quantities of blood were withdrawn to maintain the arterial pressure at 30 mm. Hg. The bleeding volume was recorded every two minutes; the respiration rate, heart rate, and rectal temperature were recorded every 10 min. This procedure was continued until one of the following criteria for reinfusion was met, at which time all of the blood in the reservoir was returned to the animal. The criteria of reinfusion used in these experiments were: (1) a reduction in the volume of blood in the reservoir at three successive two-minute intervals (automatic reinfusion), (2) a sudden drop in blood pressure followed by respiratory failure (circulatory collapse), (3) the appearance of excessive excursions in the mean blood pressure, and (4) the appearance of a 10-min, plateau in the level of blood in the reservoir (this indicated that although no further vasoconstriction was occurring, automatic reinfusion had not yet begun).

Be

M

Ai

D

M

Re

no

CO

re

D€

W

ra

ar

th

W

pe

TI

po

th

Animals which lived 48 hr. after reinfusion were considered survivors.

Results

In Series 1 of this study, consisting of 20 untreated control animals (see Table I-A), the 48-hr. mortality rate was 90%. Series 2 (Table I-B) consisted of eight animals which received chlorpromazine (5 mgm./kgm.) intravenously. In three of these animals the injection was made just before reinfusion and in five the chlorpromazine was mixed with the blood in the reservoir just before reinfusion. The mortality rate was 100%. Moreover, five of the eight treated animals died within one hour after reinfusion whereas only one of the 20 control rats died during this interval. These are significantly different (P < .01).

The table shows that there were no statistically significant differences between the control and the treated series in the prehemorrhage arterial pressure, the body weight, the duration of the hypotensive period, or in the respiratory rate, rectal temperature, and initial and maximal bleeding volumes. The arterial pressure at the end of the reinfusion was lower in the treated than in the control animals (P < .05).

TABLE I

THE EFFECTS OF CHLORPROMAZINE IN HEMORRHAGIC HYPOTENSIVE SHOCK IN RATS

	A Untreated controls	B Chlorpromazine at reinfusion
No. of rats	20	8
Body wt. (gm.)	409.5 ± 5.49*	419.5 ± 7.22
Mortality rate	90%	100%
Survival time from reinfusion of fatalities (hr.)	14.0 ± 1.9	7.0 ± 4.4
No. dying within 1 hr. after reinfusion	1/20	5/8
Arterial pressure (mm. Hg) before hemorrhage after reinfusion	141.2 ± 9.22 96.0 ± 5.78	137.4 ± 5.25 60.1 ± 14.28
Duration of hypotensive period (min.)	89.4 ± 4.39	88.5 ± 5.78
Initial bleeding volume† (ml./100 gm. body wt.)	$1.11 \pm .038$	$1.06 \pm .089$
Maximum bleeding volume (ml./100 gm. body wt.)	$2.53 \pm .063$	$2.50 \pm .167$
Respiratory rate (/min.) before hemorrhage at end of initial hemorrhage at end of hypotensive period	86.0 ± 2.47 93.2 ± 2.95 59.7 ± 2.79	78.3 ± 2.86 83.2 ± 2.84 57.5 ± 4.45
Rectal temperature (°C.) before hemorrhage at end of initial hemorrhage at end of hypotensive period	$36.3 \pm .14$ $36.0 \pm .16$ $33.6 \pm .22$	$36.3 \pm .16$ $35.9 \pm .14$ $33.4 \pm .21$

^{*}Mean and S.E.M.

Marked central depression and/or enhancement of the anesthetic was noted in the treated animals, which remained somnolent after reinfusion, in contrast to the control animals, most of which regained consciousness during reinfusion. Treated animals which did not die precipitously were followed for periods up to an hour, during which time the arterial pressure either fluctuated widely or remained constant at some level below 50 mm. Hg in spite of the rapid reinfusion. The one animal which survived 36 hr. had an average arterial pressure of 120 mm. Hg over the hour following reinfusion, but during this period many transient falls of 10–20 mm. Hg occurred. These fluctuations were also observed in the majority of the untreated rats late in the hypotensive period and in them were regarded as a sign of impending automatic reinfusion. The mechanism of these cardiovascular responses is unknown.

In the treated rats the respiration rate and rectal temperature during the post-reinfusion period continued to fall as in the hypotensive period. Two of the treated animals passed blood-stained urine, and post-mortem examination

[†] Volume of blood removed to lower blood pressure to chosen hypotensive level.

revealed that their bladders contained blood, although the kidneys appeared normal on gross examination. Blood-stained urine was not observed in any of the control animals. In all of the treated animals in which the death was observed (five of the eight) respiratory arrest preceded cardiac arrest. In the interval between the injection of chlorpromazine and death, cyanosis of the ears and feet, which was a constant feature of the late stages of the hypotensive period, appeared to decrease; this implied an improved cutaneous circulation.

Many of the fatalities in the control group convulsed frequently before death, but only one of the treated animals showed this phenomenon. It is not clear whether this is related to the reported (3) anticonvulsant activity of the drug or not.

Discussion

That chlorpromazine did have an effect on the cardiovascular system in the dosage used is indicated by the significantly lower post-reinfusion arterial pressure of the treated group and by the reduction of the cyanosis which began in the late hypotensive period and which was a regular feature of the post-reinfusion period in the control group.

It would have been surprising had chlorpromazine proved effective in preventing the onset of irreversible shock when administered so late in the hypotensive period. Dibenamine and dibenzyline, although protective when administered before or early in the shocking procedure (11, 9, 12, 1), were not helpful when injected just before reinfusion (8, 5). In fact, although certain adrenergic or ganglionic blocking agents, sympathomimetic amines, antibiotics, plasma expanders, and whole blood, have prevented or delayed irreversibility when administered early, none made any difference in the ultimate survival rate when administered late in the procedure (5).

Several statistically significant differences (e.g. in rectal temperatures, respiratory rates, maximum bleeding volumes, and duration of hypotension) are found if one compares the control rats in this study with those of Downie's (4) original series. These differences could probably be accounted for by differences in the temperature of the laboratory. The present experiments were performed during the summer months at an environmental temperature of 80.6° F. \pm 0.40 (S.E.M.) whereas the earlier series was done in the fall and winter at a temperature of 70–72° F. Other investigators (2, 10) have reported an inverse relation between survival rate and room temperature in similar experiments on dogs.

Acknowledgments

The authors are indebted to Dr. H. G. Downie for demonstrating the technique and to Prof. J. A. F. Stevenson for general criticisms and advice. The chlorpromazine used in these experiments was generously supplied by Poulenc Limited, Montreal.

References

- 1. BECK, L. and LOTZ, F. Federation Proc. 12: 12. 1953.
- 2. CLEGHORN, R. A., ARMSTRONG, J. B., and McKelvey, A. D. Can. Med. Assoc. J. 49: 355. 1943.
- 3. Courvoisier, S., Fournel, J., Ducrot, R., Kolsky, M., and Koetschet, P. Arch, intern. pharmacodynamie, 92: 305. 1953.
- 4. Downie, H. G. and Stevenson, J. A. F. Can. J. Biochem. Physiol. 33: 436. 1955.
- 5. Fine, J. New Engl. J. Med. 250: 889. 1954.

5

S e 1 e

- 6. FOURNEL, J. Compt. rend. soc. biol. 146: 561. 1952.
- 7. JAULMES, C., LABORIT, H., and BENITTE, A. Compt. rend. 234: 372. 1952.
- 8. Lotz, F., Beck, L., and Stevenson, J. A. F. Can. J. Biochem. Physiol. 33: 741. 1955.
- Remington, J. W., Hamilton, W. F., Boyd, G. G., Jr., Hamilton, W. F., Jr., and CADDELL, H. M. Am. J. Physiol. 161: 116. 1950.
- WIGGERS, C. J. Physiology of shock. The Commonwealth Fund, New York. 1950.
 WIGGERS, H. C., INGRAHAM, R. C., ROEMHILD, F., and GOLDBERG, H. Am. J. Physiol. 153: 511. 1948. Circulation, 2: 179. 1950.
- 12. ZWEIFACH, B. W., BAEZ, S., and SHORR, E. Federation Proc. 11: 177. 1952.

THE METABOLISM OF THE ERYTHROCYTE

e

b

d

b

XIII. ENZYME ACTIVITY IN THE RETICULOCYTE1

By D. Rubinstein, P. Ottolenghi, and O. F. Denstedt

Abstract

The object of the study was to ascertain the changes that occur in the activity of the enzymes of the reticulocyte during its maturation to the normocyte (adult erythrocyte). Blood specimens were taken from rabbits in which a severe anemia and a pronounced reticulosis (50–90% reticulocyte count) had been produced by giving the animals subcutaneous injections of acetylphenylhydrazine. Succinic dehydrogenase, cytochrome oxidase, and DPN-ase were found to be confined to the insoluble fraction of the cells while glucose-6-phosphate dehydrogenase and pyrophosphatase were found only in the soluble fraction (stroma-free hemolyzate). Isocitric, lactic, and malic dehydrogenases, fumarase, aconitase, and hexokinase were found to be present in both fractions. The activity of fumarase, hexokinase, and pyrophosphatase is much lower in the normocyte than in the reticulocyte while that of the isocitric, lactic, and malic dehydrogenases and of DPN-ase was of the same order in both types of cell. Aliquots of blood specimens were kept at 37° C, for 12 hr. and the activity of numerous enzymes was followed at two-hourly intervals. The enzymes which are more active in the reticulocyte undergo a diminution in activity concurrently with the decrease in the reticulocyte count. Succinic dehydrogenase, cytochrome oxidase, and aconitase are absent from the normocyte. The significance of these changes with respect to the maturation of the reticulocyte is discussed.

Exposure of hemolyzates of the blood specimens to the enzyme ribonuclease, to destroy any ribonucleic acid that may be present, did not alter the activity of any of the enzymes tested. The DPN-ase of the reticulocyte, as in the normocyte, was found to be a nucleosidase which splits the linkage between nicotinamide and ribose.

Introduction

It has long been known that the reticulocyte has an active aerobic metabolism (28) ($-Q_{\rm O_2}=1.0$ (30)). The respiratory activity is inhibited by cyanide and by malonate. Sherwood Jones *et al.* (27) observed that fluoroacetate decreases the oxygen uptake and favors the accumulation of citrate. Borsook *et al.* (4) have shown that the reticulocyte can incorporate amino acids into protein.

The mature mammalian erythrocyte, on the other hand, shows insignificant, if any, respiratory activity. It is incapable of utilizing pyruvic acid, forming citric acid (22), or incorporating amino acids into protein (4). It is unlikely that the cell obtains any energy from aerobic metabolism, and compared with the reticulocyte it is much less active in its production and utilization of energy.

It is generally believed that the reticulocyte is a precursor of the erythrocyte in hemopoiesis. This inference is based on the well-substantiated observation that the reticulocyte count rises in animals made progressively more anemic

¹Manuscript received October 25, 1955.

Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec, with the financial assistance of Defence Research Board of Canada, Grant No. 9350-01, Project D 50-93050-01.

The following abbreviations have been used throughout the paper: ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; DPN-ase, diphosphopyridine nucleotidase; O.D., optical density; RNA, ribonucleic acid: SFH, strong-free hemolyzate; TPN, triphosphopyridine nucleotide; TPN.H, reduced TPN; WH, whole hemolyzate.

either by repeated blood removal or by intravascular destruction of the red blood cells (for example by repeated subcutaneous injection of phenylhydrazine or *Plasmodium berghei*). The progressively increased concentration of reticulocytes has been shown to account for a corresponding increase in the concentration of RNA in the blood under these conditions (5), and also an increase in the rate of incorporation of amino acids into the proteins of the cells (14, 11). The presence of RNA in the reticulocyte is a feature which distinguishes the latter cell from the erythrocyte. The RNA is believed to be the material in the reticulocyte that gives the characteristic staining with brilliant cresyl blue (10). Furthermore it is known that the rate of decrease in the content of RNA in the blood runs parallel with the rate of maturation (disappearance) of the reticulocytes. The maturation time *in vivo* appears to be variable, ranging from 8 to 140 hr. (3, 31). Heath and Daland (9) found that the rate of maturation *in vitro* at 37° C. corresponds with that noted *in vivo*.

The present investigation was undertaken to compare the metabolic activity of the mature red blood cell with that of the reticulocyte and to ascertain the enzymological changes that occur as the reticulocyte matures.

Methods

Blood

Rabbits were made anemic by giving them daily subcutaneous injections of 25 mgm. acetylphenylhydrazine in alcoholic (50%) solution for a period of six days. On the eighth day, about 30 ml. of blood was withdrawn from the marginal ear vein into a syringe containing heparin. The hematocrit and the hemoglobin concentration in the specimens were found to be greatly diminished and the proportion of reticulocytes ranged from 50 to 95% of the total number of red blood cells. Washed cell specimens were prepared by suspending the cells in cold isotonic KCl and centrifuging the samples. The procedure was repeated four times, and each time the supernatant "washings" were discarded. When hemolyzates were required, the washed cells were hemolyzed by alternate freezing (in a bath containing dry ice and alcohol) and thawing, the operation being repeated three times. The particulate fraction was separated by centrifuging the hemolyzate at 3000 r.p.m.

Other Preparations

TPN was prepared from hog livers according to the method of LePage and Mueller (15), and the barium salt of dl-isocitrate, by the method of Fittig $et\ al.$ (7). Other preparations used were commercial products. The substrates were used in the form of sodium or potassium salts, and, when necessary, the pH of the solutions was adjusted to 7.2.

Analyses

f

The succinic, malic, lactic, and isocitric dehydrogenases were estimated by the ferricyanide technique of Quastel and Wheatley (20). Cyanide was added to all the flasks, with the exception of those used in the determination of succinic dehydrogenase, in order to fix the keto-acids formed in the reactions. The ultraviolet absorption at 340 m μ by the TPN.H formed in the reaction was used as a measure of the glucose-6-phosphate dehydrogenase activity. Aconitase was estimated by incubating the cells with citrate and phosphate buffer, deproteinizing the solution, and measuring the absorption of the protein-free filtrate at 240 m μ (23). Fumarase was assayed spectrophotometrically by the method of Racker (21). Cytochrome oxidase was determined manometrically, using p-phenylenediamine as the reducing agent (24). Hexokinase² was determined by measuring the rate of disappearance of glucose at 37° C. in blood containing added ATP, Mg⁺⁺, and fluoride. Pyrophosphatase² activity was measured by the rate of liberation of inorganic phosphate by the action of SFH on pyrophosphate in the presence of Mg⁺⁺ at 37° C.

Glucose was estimated by the method of Nelson (16) and phosphate by the method of Fiske and SubbaRow (6). RNA was assayed by Schneider's procedure (26), and DPN, either by means of alcohol dehydrogenase or by the reaction with sodium cyanide (12). The two methods for the determination of DPN gave comparable results. Reticulocyte counts were done on blood smears by the 'dry' method (29). The proportion (percentage) of reticulocytes was estimated by counting 1000 red cells in several fields of duplicate smears.

ca.

D

pl

iso

gr

la

m

ce

as

SU

er

ni

C

de

m

a

of

of

b

Results

The oxygen uptake of washed reticulocytes from rabbit blood was studied, both in the presence and in the absence of the various substrates. The results are given in Table I.

The control specimen (without added substrate) gave a $-Q_{0_1}$ of 1.0. With succinate as the added substrate a marked increase in the oxygen uptake was obtained. The addition of malonate reduced the oxygen uptake with succinate to about 25% of the former value. Malonate caused a similar inhibition of the endogenous oxygen uptake, thus suggesting that the oxidation mechanism involves succinic dehydrogenase. With glucose, lactate, or malate as the added substrate only a small increase in the oxygen uptake occurred. Nucleated (chicken) erythrocytes, under the various conditions described, behaved in a comparable manner (23). The inhibition of oxygen uptake by fluoride, observed with or without added glucose, suggests that the glycolytic system through to the production of pyruvate is involved in the aerobic metabolism of the reticulocyte.

The distribution of certain enzymes between particulate and soluble fractions of reticulocytes also was investigated. Attention was given particularly to the enzymes involved directly or indirectly in the energy-yielding mechanism of the cell. The results are given in Table II.

²We wish to thank Dr. S. Kashket for assistance in carrying out the hexokinase determinations, and Dr. A. Malkin for performing the pyrophosphatase assays.

TABLE I

Effect of added substrates and inhibitors on the respiration of the reticulocyte

Substrates and inhibitors	$-Q_{O2}$	% change
None	1.0	
Malonate	0.24	-76
Succinate	1.97	+97
Succinate + malonate	0.49	-75*
Fluoride	0.37	-63
Glucose	1.24	+24
Glucose + fluoride	0.37	-67*
dl-Lactate	1.30	+30
Malate	1.12	+12

^{*}The controls for these experiments contained only the substrate.

FLASK CONTENTS: Flasks contained 1 ml. washed cells, made up to a total volume of 2.5 ml. with Krebs-Ringer-phosphate buffer, pH 7.4. Final concentration of substrate was 0.1 M in all cases. Final malonate and fluoride concentration was 0.025 M. 0.2 ml. 20% KOH was placed in center well. Gas phase: air. Temperature: 37.5° C.

It is apparent that cytochrome oxidase, succinic dehydrogenase, and DPN-ase are associated entirely with the particulate fraction. Pyrophosphatase and glucose-6-phosphate dehydrogenase, on the other hand, are found only in the particle-free hemolyzate. The enzymes lactic, malic, and isocitric dehydrogenases, aconitase, fumarase, and hexokinase were found in both fractions. In all these cases, except with aconitase, the activity was greater in the particle-free hemolyzate.

It may be observed in Table II that the sum of the activities of the particulate fraction and of the particle-free hemolyzate, in the case of lactic and malic dehydrogenases, was greater than the activity of the unfractionated cells. The result was consistently obtained in repeated experiments. To ascertain whether this might be attributable to the action of a nucleosidase such as DPN-ase, which is known to be present in the stroma of the rabbit erythrocyte (1), another series of experiments was carried out with added nicotinamide. The latter inhibits the stroma DPN-ase of the rabbit erythrocyte (2). The results are given in Table III.

It is evident that the presence of nicotinamide markedly decreased the destruction of DPN by the stroma. Increasing the concentration of nicotinamide or decreasing the proportion of blood did not decrease the total of the activities. The lower results obtained in the presence of the stroma could not be attributable to the DPN-ase activity of the latter because inhibition of the DPN-ase with various amounts of nicotinamide did not alter the sum of the activities.

A pronounced reticulocytosis was produced in rabbits, the reticulocyte count ranging from 55 to 82% of the total cell count. In order to obtain a better comparison of the activity of the various enzymes the mean of the values obtained for each was multiplied by a factor to give the activity which

TABLE
DISTRIBUTION OF VARIOUS ENZYMES

A!

T

b;

D

n

b

tl

p

a

n

tl

m

re

N

b

Enzymes, etc.	Reticulocyt count of sample		Whole hemolyzate activity (Q)
Malic dehydrogenase	55	μl. CO ₂ /mgm./hr.	8.6
Lactic dehydrogenase	72	μl. CO ₂ /mgm./hr.	10.1
Isocitric dehydrogenase	72	μl. CO ₂ /mgm./hr.	2.7
Succinic dehydrogenase	60	μl. CO ₂ /mgm./hr.	1.9
Glucose-6-phosphate dehydrogenase	57	Δ O.D./mgm./hr.	1.1
Cytochrome oxidase	60	μl. O ₂ /mgm./hr.	-3.1
Aconitase	81	Δ O.D./mgm./hr.	0.02
Fumarase	55	Δ O.D./mgm./hr.	0.27
Hexokinase	50	μM. glucose uptake/mgm./hr.	0.17
DPN-ase	81	μM. DPN destroyed/mgm./hr	. 0.46
Pyrophosphatase	81	μM. PO ₄ produced/mgm./hr.	5.20
Ribonucleic acid	57	μgm./ml. blood	733

^{* %} of whole hemolyzate.

FLASK CONTENTS: Glucose-6-phosphate dehydrogenase.—Each flask contained 0.02 ml. enzyme, 0.04 M phosphate buffer, pH 7.4, 2.6 \times 10⁻² M glucose-6-phosphate, 1 \times 10⁻⁴ M TPN, 0.04 M nicotinamide. Volume made up to 2.5 ml. with isotonic KCl.

Malic dehydrogenase.—Each flask contained 0.5 ml. enzyme preparation, 1.5 × 10-2 M NaHCO₂, 0.2 ml. of 11% K₃Fe(CN)₆ neutralized with bicarbonate, 0.04 M nicotinamide, 3 × 10⁴ M DPN, 0.1 M. sodium *l*-malate, and 0.2 ml. of 0.65 M NaCN neutralized with HCl. Total volume made up to 2.5 ml. with isotonic KCl. Gas phase 95% N₂: 5% CO₂. Temp. 37.5° C.

Lactic dehydrogenase.—Conditions as for malic dehydrogenase, except that $0.1\ M$ sodium dl-lactate was used instead of malate.

Isocitric dehydrogenase.—Each flask contained 0.5 ml. enzyme preparation, 0.2 ml. K₃Fe(CN)₆ prepared as above, 0.04 M sodium dl-isocitrate, 10-1 M TPN, 0.2 ml. neutralized NaCN, prepared as above, 0.04 M nicotinamide. Volume made up to 2.5 ml. with isotonic KCl. Gas phase 95% N₂: 5% CO₂. Temp. 37.5° C. Succinic dehydrogenase.—Each flask contained 1.6 ml.

Succinic dehydrogenase.—Each flask contained 1.0 ml. enzyme preparation, 0.1 M sodium succinate, 0.2 ml. K_3 Fe(CN)₆, 1.54 \times 10-2 M NaHCO₃. Made up to total volume of 2.5 ml. with isotonic KCl. Gas phase 95% N_2 : 5% CO₂. Temp. 37.5° C.

would theoretically have been obtained if the blood samples had been made up entirely of reticulocytes. In making this adjustment the assumption was made that reticulocytes and erythrocytes have approximately the same dry weight. The reticulocyte actually is slightly larger (19) and has a lower specific gravity (13) than the erythrocyte. The error introduced by the adjustment of the values probably is small.

The enzymes listed in Table IV may be divided into two groups. Group 1 includes the lactic, isocitric, and glucose-6-phosphate dehydrogenases and DPN-ase, which are found in both the reticulocyte and the mature erythrocyte and have approximately the same degree of activity. The ratios between the respective activities in the two types of cell were less than two. The number of samples available was not sufficient to enable us to establish whether there was a significant difference in the activities. Furthermore, the differences in dry weight and cell counts, and the individual variation between the animals

2.

n ı

d

c n 1.

e S

y

r

e

1

d

te

ıe

er

re

in

ls

AND RNA OF THE RETICULOCYTE

Stroma-free hen	nolyzate-activity	Particulate for	raction-activity	Total activity
(Q)	(% activity)	(Q)	(% activity)	for (%*)
8.6	90	46	56	146
18.4	166	44.5	32	198
1.8	60	12.3	33	93
0.1	8	22.5	110	118
0.94	85	0	0	85
-0.2	6	-30.1	89	95
0.01	43	0.18	73	116
0.21	68	0.55	21	89
0.12	68	0.23	12	80
0.00	0	4.45	91	91
4.24	79	0.4	1	80
505	69	222	30	99

Cytochrome oxidase.—Each flask contained 0.4, 0.8, or 1.2 ml. of enzyme preparation, $6\times 10^{-6}~M$ cytochrome c, 0.07 M phosphate buffer, pH 7.2, 0.3 M p-phenylenediamine, 0.2 ml. 20% KOH in center well. Volume made up to 3.0 ml. with isotonic KCl. Gas phase: air. Temp. 37.5° C

Aconitase. - Each flask contained 1.5 ml. of the enzyme preparation, 0.06 M phosphate buffer, pH 7.2, 0.08~M sodium citrate. Total volume was made up to 4.0 ml. with isotonic saline. Incubation time 40 min. Temp. 37.5° C. Preparation, after incubation, was deproteinized by boiling.

Fumarase.—Each cuvette contained 0.02 ml. enzyme preparation, 0.08 M sodium malate, 0.05 M phosphate buffer, pH 7.2. Made up to total volume of 3.0 ml. with saline

DPN-ase.—Each flask contained 0.06 ml. enzyme preparation, DPN (1.5 μM./ml. final solution), 0.04 M phosphate buffer, pH 5.0, made up to volume of 1.0 ml. with saline. Incubation time 15 min. Temp. 37.5° C. Solutions were deproteinized by boiling. The remaining DPN was analyzed by the cyanide or the alcohol dehydrogenase method.

Pyrophosphalase.—Each flask contained 10-3 M pyrophosphate, 0.02 M MgCl₂, and 0.1 ml. enzyme preparation (dialyzed for two and one-half hours against running tap water and diluted 1:2 with distilled water) and made up to volume of 2.0 ml. with 0.1 M 'Tris' buffer, pH 7.5. Incubation time 15 min. Temp. 37.5° C.

Hexokinase.—Each flask contained 600 μ gm. glucose, $1.3 \times 10^{-2}~M$ phosphate buffer and $\times~10^{-2}~M$ glycylglycine buffer, both at pH 8.0, $6 \times 10^{-2}~M$ MgCl₂, $1 \times 10^{-2}~M$ NaF, $\times~10^{-3}~M$ sodium ATP, and 0.2 ml. of enzyme preparation. Incubation time 60 min. Temp. 37.5° C.

made it practically impossible to tell whether there is a significant difference between the behavior of the two types of cell with respect to the activity of the enzymes of group I (Table IV).

The second group of enzymes includes fumarase, hexokinase, pyrophosphatase, succinic dehydrogenase, cytochrome oxidase, and aconitase, which are present in the reticulocyte but either absent or much less active in the mature erythrocyte. The ratio of the activities of these enzymes between the reticulocyte and the erythrocyte is 13:1 or greater. The first two enzymes mentioned are present in both types of cell but are much more active in the reticulocyte. The last three enzymes are present only in the reticulocyte. Malic dehydrogenase appears to be intermediate between the two groups, being about three times as active in the reticulocyte as in the erythrocyte.

TABLE III EFFECT OF NICOTINAMIDE ON THE DESTRUCTION OF DPN BY DPN-ASE

Nicotinamide	Method of DPN assay	μM. DPN destroyed/ml./hr.	% destroyed
-	Alcohol dehydrogenase	41.5	92
-	Cyanide	42.0	93
+	Alcohol dehydrogenase	5.0	11
+	Cyanide	5.2	12

FLASK CONTENTS: as in Table II. Whole hemolyzate was used as the enzyme preparation. Nicotinamide concentration was 0.06 molar.

PER CENT OF ORIGINAL ACTIVITY

ret

hr De in th

th hi co di re

py

pe D

th

In view of these differences further experiments were carried out to follow the progressive change in the activity of the various enzymes during maturation of the reticulocytes in vitro. Blood specimens containing a high proportion of reticulocytes were incubated at 37° C. with added glucose (final concentration 0.05 M) and penicillin. Small samples were withdrawn at intervals of two hours for 12 hr. for assay of the enzymes. Our findings confirmed the observation of Nizet (17) that the reticulocytes mature more rapidly in the presence of glucose. The results of four such experiments are given in Table V and Figs. 1-4.

TABLE IV COMPARISON OF ENZYME ACTIVITIES OF THE NORMOCYTE AND THE RETICULOCYTE

	Prepar-				Reticul	ocytes				Normo	cyt	es	Reticulocyte
Enzyme	ation	N^*	Act	ua	Qt	Corre	ect	ed Q‡	N		0		Normocyte
DPN-ase	Stroma	5	5.2	±	1.5	5.2	±	1.5	3	4.9			1.1
Lactic dehydrogenase	SFH	5	14.7	±	4.1	17.7	±	4.1	4	10.3	±	2.5	1.7
Glucose-6-phosphate dehydrogenase	SFH	2	1.02			1.25			2	0.68			1.8
Isocitric dehydrogenase	WH	4	2.3	±	0.2	3.0	±	0.1	4	1.6	±	0.3	1.9
Malic dehydrogenase	SFH	6	6.8	±	2.2	9.1	±	2.4	4	3.4	±	0.3	2.7
Fumarase	WH	7	0.44	±	0.17	0.66	±	0.14	4	0.05	±	0.01	13.2
Hexokinase	WH	4	0.26	±	0.03	0.41	±	0.11	4	0.03	±	0.01	13.7
Pyrophosphatase	SFH	3	4.5			5.8			2	0.3			19.3
Succinic dehydrogenase	Stroma	4	19.1	±	4.3	33.0	±	3.4	3	0			00
Aconitase	WH	2	0.02	1		0.02	4		2	0			00
Cytochrome oxidase	Stroma	4	-3.1	±	1.0	-5.5	±	2.1	3	0			00

* N = Number of samples.

† Units of Q values: as described in the discussion of the individual enzymes.

‡ Q corrected to 100% reticulocytes.

§ S.D., when given. Calculated for enzymes having more than three samples. Others, arithmetic mean.

FLASK CONTENTS: as in Table II.

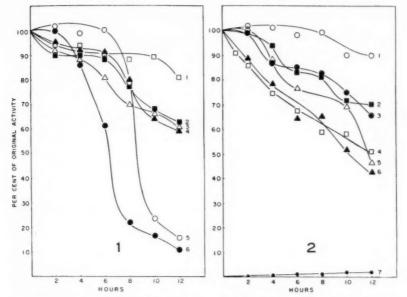


FIG. 1. Change in enzyme activity of the reticulocyte during incubation at 37° C. Original reticulocyte count: 94%.

Legend: 1. Reticulocyte count

rs,

2. Fumarase

Succinic dehydrogenase
 Hexokinase

5. Pyrophosphatase

6. Aconitase

FIG. 2. Change in enzyme activity of the reticulocyte during incubation at 37° C. Original reticulocyte count: 43%.

Legend: 1. Lactic dehydrogenase

2. Fumarase 3. Hexokinase

4. Reticulocyte count

Cytochrome oxidase

Succinic dehydrogenase
 Spontaneous hemolysis

Table V indicates the activity remaining in the samples at the end of 12 hr., expressed as percentage of the activity at the beginning of the incubation period. It is obvious that the rate of maturation (as measured by the decrease in reticulocyte count) differed considerably in the four experiments. In run 1 the reticulocyte count fell only from 94 to 81% during the 12-hr. period, while in runs 2 and 4 a much greater decrease was observed. The failure of the reticulocytes to mature in blood specimens in which the count was very high was observed also in other experiments. The ribonucleic acid (RNA) content of the samples was followed. Under these conditions the rate of disappearance of the RNA appears to be unrelated to the rate of fall in the reticulocyte count.

Hexokinase, aconitase, succinic dehydrogenase, cytochrome oxidase, and pyrophosphatase showed a marked decrease in activity during the 12-hr. period of incubation. The fumarase activity usually decreased, while that of DPN-ase and the lactic and malic dehydrogenases remained unchanged throughout the experimental period. Figs. 1–4 illustrate the progressive

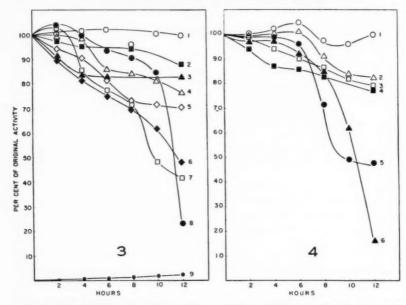


Fig. 3. Change in enzyme activity of the reticulocyte during incubation at 37° C. Original reticulocyte count: 74%.

Legend: 1. Malic dehydrogenase
2. Lactic dehydrogenase

3. Fumarase 4. Hexokinase

5. Reticulocyte count 6. Aconitase

Succinic dehydrogenase
 Pyrophosphatase

9. Spontaneous hemolysis

FIG. 4. Change in enzyme activity of the reticulocyte during incubation at 37° C. Original reticulocyte count: 60%.

Orig

Fina RN.

Hen

Aco

Suce Fun Cvt

Pyr

Lac

Mal DP

enz

the

con

to

act

wei

enz

whi

mo

the

Suco

Cyt

Rib

F

9

Legend: 1. Lactic dehydrogenase

DPN-ase
 Malic dehydrogenase

4. Fumarase

Cytochrome oxidaseReticulocyte count

changes in the enzyme activities during the period. The curves correspond with the results given in Table V. It is evident that there is no relationship between the rate of decrease in activity of various enzymes in any of the experiments. Thus the aconitase activity, indicated in Figs. 1 and 3, began to fall almost immediately while pyrophosphatase activity remained unchanged for many hours before it began to decrease. In view of the variability in the behavior of the enzymes in the four experiments it is impossible to say which of the enzymes is the first to undergo diminution during maturation of the cell. The decrease in the activity of the enzymes did not parallel the decrease in reticulocyte count. However, while the rate of maturation (diminution in the number of reticulocytes) was greater in some specimens than in others (e.g. Fig. 1) a decrease in the activity of all the enzymes tested, with the exception of lactic and malic dehydrogenases, occurred in all instances. It may be noted also that the rate of decrease of the activity of the various

TABLE V

EFFECT OF INCUBATION ON THE ENZYME ACTIVITY OF RETICULOCYTES

		Run n	umber	
	1	2	3	4
Original count (%)	94	43	74	60
Final count (%) at end of incubation period	81	23	53	10
RNA (%) of original at end of incubation period	87	75	71	
Hemolysis (%) at end of the experimental period	2	2	2	1
Enzyme system	Per	cent of the	original act	ivity
Hexokinase	60	66	75	_
Aconitase	11	-	48	-
Succinic dehydrogenase	61	43	42	-
Fumarase	63	70	8.3	79
Cytochrome oxidase	-	47		47
Pyrophosphatase	16	-	22	-
Lactic dehydrogenase	_	90	87	100
Malic dehydrogenase	-	_	100	80
DPN-ase			-	81

enzymes, as indicated in the curves in Figs. 1-4, showed no correlation with the magnitude of the proportion of reticulocytes in the original samples.

Since the reticulocyte count is merely a measure of the number of cells containing stainable material (presumably RNA), an experiment was designed to determine what role, if any, RNA plays in the regulation of the enzyme activity. Hemolyzates of blood containing a high percentage of reticulocytes were incubated for two hours with ribonuclease and the activity of several enzymes was compared with the activity in another aliquot of the same blood, which was incubated without ribonuclease. Cytochrome oxidase, succinic dehydrogenase, and fumarase were studied, since these enzymes are much more active in the reticulocyte and decrease in activity or disappear from the cell during maturation. The results are shown in Table VI.

TABLE VI EFFECT OF RIBONUCLEASE ON ACTIVITY OF ENZYMES

d

e

e n rs ie It

Enzymes, etc.	Preparation used	Units	With ribonuclease	Control
Succinic dehydrogenase	Stroma	μl. CO ₂ /hr.	83	41
Fumarase	SFH	Δ O.D./30 min.	0.775	0.790
Cytochrome oxidase	Stroma	μl. O ₂ /hr.	-29	-34
Ribonucleic acid	WH	µgm./ml. blood	83	950

FLASK CONTENTS: as in Table II. 0.04 ml, blood was used for the fumarase experiment.

w

tie

to

th

ar

lit

la

CC

st

CC

m hi

de

th

CC

la

CC

to

bl

st

gr

th

th

CE

in

de

of

fr

fo

ch

re

bi

fr

T

in

fo

m

n

C

W

The added ribonuclease did not greatly alter the fumarase or cytochrome oxidase activity, although more than 90% of the RNA had undergone hydrolysis. However, the succinic dehydrogenase activity was increased after the action of the added enzyme. This is contrary to Zittle's observation (32) that ribonuclease decreases the succinic dehydrogenase activity of rat liver. The decrease may possibly have been due to the presence of a proteolytic enzyme contaminant in the preparations of the ribonuclease available at that time. Our result with the reticulocytes may be attributable to an unmasking of the succinic dehydrogenase by the action of the enzyme. This phenomenon is being studied further. However, the figures in Table VI indicate that the destruction of the ribonuclease was not responsible for the disappearance of or decrease in the activity of the enzymes in question.

Discussion

The results of this study confirm that the reticulocyte has a more complete and a more active metabolism than the mature mammalian erythrocyte. In contrast with the latter cell, which is incapable of respiratory activity and depends upon the glycolytic system for its energy requirements, the reticulocyte possesses the tricarboxylic-acid and the cytochrome systems. Its respiratory activity is decreased in the presence of poisons such as fluoride and malonate, but is increased in the presence of added succinate. Cytochrome oxidase, succinic dehydrogenase, and aconitase are absent from the mature erythrocyte. Fumarase, hexokinase, and pyrophosphatase are present in both types of cell but are much less active, that is, are present in much smaller concentrations, in the erythrocyte. Enzymes, including DPN-ase and the lactic and glucose-6-phosphate dehydrogenases, which are not directly involved in the tricarboxylic acid cycle, are about equally active in the reticulocyte and the erythrocyte. Contrary to expectation isocitric dehydrogenase also is about equally active in the two types of cell. Apparently this enzyme is quite stable and water-soluble and thus is not destroyed during the maturation of the reticulocyte. The reticulocytosis which develops in certain types of anemia accounts for the observation by former workers that the activity of certain enzymes is increased in the blood as animals become more anemic.

The study of the change in the activity of various enzymes during the incubation of specimens of blood containing a high proportion of reticulocytes affords the advantage that one can study the behavior of a fixed population of cells. Thus if the activity of any enzyme is observed to decrease the alteration cannot be attributed to the removal from the circulation of cells containing the enzyme, or to the entrance of cells which lack it. Either or both of these conditions may possibly complicate the picture in fresh specimens taken over a period of time. Thus the decrease in the observed specific activity must be attributable to a change within the cells in the specimen.

The experiments show clearly that the rate of maturation during the incubation of reticulocytes *in vitro* at 37° C. is variable. As a rule, the higher the proportion of reticulocytes in the specimen (induced by red cell destruction

le

16

n

of

e-

le

n

115

n-

1e

te

e.

ty

15.

de

ne

ire

in

ler

he

tly

u-

ise

me

ra-

oes

of

.

he

tes

ion

the

ells

or

eci-

ific

the

her

ion

with acetylphenylhydrazine in the rabbit), the slower was the rate of maturation. Further, since the stainable granules in the reticulocyte are supposed to be composed largely, if not entirely, of RNA one might, at first thought, expect to find a correlation between the rate of maturation, as indicated by the rate of disappearance of reticulocytes in the specimen during incubation, and the rate of disappearance of RNA from the cells. Our results indicate little or no correlation. A closer examination of the findings explains the The progressive decrease in the reticulocyte count was lack of correlation. considered to indicate the rate of maturation and all the cells containing stained granules, regardless of the quantity of the stainable material, were counted as reticulocytes. Obviously these cells represent various stages of High reticulocyte counts in fresh blood specimens reflect a higher rate of production of these immature cells in the bone marrow than do lower counts. Similarly, the mean degree of maturation of the reticulocytes is probably lower in specimens with high counts. During the maturation of the cells the RNA content is progressively decreased, but all the cells that contained any stained material were counted. It is thus possible to have a large decrease in the RNA content with a relatively small decrease in the count. It is possible also that some of the maturing cells may contain only a small quantity of RNA which may disappear without greatly altering the total amount of RNA in the sample. Variation in the amount of stainable material in the cells which constitute the popu'at on of the reticulocytes in a blood specimen has been noted by Nizet and Robscheit-Robbins (18).

For convenience of discussion, the enzymes of the reticulocyte that were studied in the present investigation were divided into two groups, namely, group 1, the enzymes which are about equally active in the two types of cell, that is, which do not suffer a decrease in concentration during maturation of the reticulocyte, and group 2, those that are present in much higher concentration in the reticulocyte than in the erythrocyte. During the 12-hr. incubation treatment the enzymes of group 2 (cf. Table V) underwent a decrease in activity ranging from 30 to 80%. These enzymes in other types of somatic cell, for example liver, are generally associated with the particulate fraction in the cell. Pyrophosphatase is an exception in that it is generally found in the cytoplasm. The enzymes of group 1 apparently undergo little change in activity during maturation of the reticulocyte. Although the reticulocyte contains neither a nucleus nor mitochondria, the mode of distribution of the enzymes in the reticulocyte between particulate and the soluble fractions of the cell is consistent with the distribution in other somatic cells. The enzymes may be divided into two classes: (1) the 'soluble' ones which, in other somatic cells, occur in the cytoplasm in water-soluble or extractable form associated with the solid constituents of the cell such as the nucleus, mitochondria, etc., or in the cell membrane; (2) the 'insoluble' ones which do not occur in the cytoplasm and which remain attached to the particulate constituents of the cell. Succinic dehydrogenase and cytochrome oxidase, which are of the insoluble type of enzyme, in these as in other cells, are associated with the particulate fraction. Malic and isocitric dehydrogenases, aconitase, and fumarase, on the other hand, which are of the soluble type of enzyme and are usually associated with the mitochondria in other somatic cells, occur mainly in the soluble cytoplasmic fraction of the reticulocyte but a substantial proportion of these enzymes (equal to 25–50% of the activity of the whole hemolyzate) is present also in the particulate fraction. It may be, however, that the particulate material of the intact cell contains a higher proportion of the enzymes and that they undergo partial detachment and solubilization during the hemolysis of the cells. The retention of a substantial portion of the enzymes by the particulate matter despite the repeated washings of the material leaves little doubt that they, at least in part, are naturally associated with the insoluble material. In contrast with the 'insoluble' group the enzymes of the 'soluble' group, not normally found in mitochondria of somatic cells, occur entirely in the cytoplasmic fraction (SFH) of the reticulocyte.

From our studies and those of others on the development of the mammalian red blood cell it would appear that the history of the cell, from its most primitive form in the bone marrow to the mature erythrocyte, consists of a sequence of changes in which the metabolic activity of the cell undergoes a rapid and progressive diminution. In the earlier stages of its hurried development the cell has a nucleus and doubtless contains mitochondria. During the early and rapid stage of development, RNA disappears concurrently with the formation of hemoglobin. The loss of the mitochondria probably takes place at an earlier stage or concurrently with the loss of the nucleus. With these changes the respiratory capacity of the cell is lost and the metabolic activity is greatly diminished. It is by virtue of the pronounced diminution in metabolic activity that the life span of the mature erythrocyte is prolonged to 125 days. It may be that during the history of the red cell the metabolic activity falls off in a logarithmic fashion and that the progressive diminution during the aging of the mature erythrocyte is but a continuation of a process of diminishing energy metabolism that has gone on from the earliest stages. The ultimate failure of the erythrocyte at the end of its life span may possibly be determined by the inability of the cell to maintain its energy requirement. This is supported by the observations that the addition of adenosine to preserved blood specimens improves the viability of the cells (8) by providing a utilizable source of energy in the form of ribose phosphate which is metabolized to lactate and yields ATP (25).

References

1. ALIVISATOS, S. G. A. and DENSTEDT, O. F. Science, 114: 281. 1951.

2. ALIVISATOS, S. G. A. and DENSTEDT, O. F. J. Biol. Chem. 199: 493. 1952.

3. BAAR, H. S. and LLOYD, T. W. J. Physiol. 98: 12P. 1940.

4. Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H. J. Biol. Chem. 196: 669. 1952.

5. Burt, N. S., Murray, R. G. E., and Rossiter, R. J. Blood, 6: 906. 1951.

6. FISKE, C. H. and SUBBAROW, Y. J. Biol. Chem. 66: 375. 1925.

7. FITTIG, R. and MILLER, H. E. Ann. Chem. 255: 43. 1889.

ases, pe of natic

but ivity may igher

and ntial nings

rally roup ia of ticu-

alian most of a

elopuring ently oably cleus. bolic

ution inged bolic ution ocess ages. ssibly nent. ne to iding

abol-

8. Gabrio, B. W. and Huennekens, F. M. Federation Proc. 14: 217. 1955.

9. HEATH, C. W. and DALAND, G. A. Arch. Internal Med. 46: 553. 1930.

10. HEATH, C. W. and DALAND, G. A. Arch. Internal Med. 48: 133. 1931.

11. HOLLOWAY, B. W. and RIPLEY, S. H. J. Biol. Chem. 196: 695. 1952.

12. KAPLAN, N. O., COLOWICK, S. P., and NASON, A. J. Biol. Chem. 191: 473. 1951.

13. Key, J. A. Arch. Internal Med. 28: 511. 1921.

14. Koritz, S. B. and Chantrenne, H. Biochim. et Biophys. Acta, 13: 209. 1954.

15. LEPAGE, G. A. and MUELLER, G. C. J. Biol. Chem. 180: 975. 1949.

16. Nelson, N. J. Biol. Chem. 153: 375. 1944.

17. NIZET, A. Compt. rend. soc. biol. 140: 1076. 1946.

18. NIZET, A. and ROBSCHEIT-ROBBINS, F. S. Blood, 5: 648. 1950.

19. Persons, E. L. J. Clin. Invest. 7: 615. 1929.

20. QUASTEL, J. H. and WHEATLEY, A. H. M. Biochem. J. 32: 936. 1938.

21. RACKER, E. Biochim. et Biophys. Acta, 4: 211. 1950.

22. RUBINSTEIN, D. Ph.D. Thesis, McGill University, Montreal, Que. 1952.

RCBINSTEIN, D. and DENSTEDT, O. F. J. Biol. Chem. 204: 623. 1953.
 RUBINSTEIN, D. and DENSTEDT, O. F. Can. J. Biochem. Physiol. 32: 548. 1954.

 RUBINSTEIN, D., KASHĶET, S., and DENSTEDT, O. F. Can. J. Biochem. Physiol. 34: 61. 1956.

26. SCHNEIDER, W. C. J. Biol. Chem. 161: 293. 1945.

SHERWOOD JONES, E., MAEGRAITH, B. G., and GIBSON, Q. H. Ann. Trop. Med. Parasitol. 47: 431. 1953.

28. WARBURG, O. Z. physiol. Chem. 59: 112. 1909.

 WHITBY, L. E. H. and BRITTON, C. J. C. Disorders of the blood. 8th ed. The Blakiston Co., Philadelphia. 1946.

30. WRIGHT, G. P. J. Gen. Physiol. 14: 201. 1930.

31. YOUNG, L. E. and LAWRENCE, J. S. J. Clin. Invest. 24: 554. 1945.

32. ZITTLE, C. A. J. Biol. Chem. 162: 287. 1946.

SPECTROPHOTOMETRIC ANALYSIS OF PROTEINS BY A SELECTIVE FILTER TECHNIQUE:

By E. Annau

Abstract

Ultraviolet absorption spectra of characteristic shapes and vibrational fine structures have been produced of serum albumin, ovalbumin, casein, and gelatin. This was achieved by a method in which a standard reference solution of an appropriate xanthine concentration was used as an optical filter for the spectrophotometric measurements.

Introduction

The ultraviolet spectra of many proteins are distinguished through a selective absorption in the region at 250 to 300 m μ due to their aromatic amino acid residues. The vibrational fine structure of the aromatic chromophores is, however, obscured and shifted toward the longer wave lengths by the interplay of the chemical bondages and various physical factors such as the Stark and Zeemen effect (8) within the protein molecule. The difficulty in the differentiation of the fine structures of the proteins was eventually overcome by the low temperature technique initiated by Lavin and Northrop (7) and later by the moving-plate method of Holiday (6).

There is also evidence on the other hand that protein hydrolyzates or adequately composed amino acid mixtures show absorption spectra which are to some extent comparable to those of undergraded proteins (9). This means from a spectrophotometrical point of view that the protein molecule might be also sufficiently characterized by the relative amount of its constituents and thus considered as a mixture. It was shown in previous communications (1, 3) that a spectrophotometric identification of individual components in mixed solutes could be achieved by the use of a standard reference solution of a suitable composition, acting as an optical filter in the Beckman DU spectrophotometer. Considering the composite nature of proteins, it was assumed that the application of a similar filter technique might effect some degree of resolution in the vibrational fine structure of the protein components, thus making their spectrophotometric identification possible.

The present study is concerned with these problems.

Materials and Methods

The following 0.1% solutions of commercial pure protein preparates were used for the measurements at pH 7.3 and pH 10.0: crystalline bovine serum albumin (Armour), crystalline egg albumin (Armour), casein (Fisher Reagent). The concentration of the gelatin (Difco) solution employed was 0.3%, because of its low molecular extinction.

¹Manuscript received November 17, 1955. Contribution from Animal Pathology Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec. The standard reference solution was freshly diluted daily from a stock solution containing 15.2 mgm. xanthine per 100 ml. $0.01\ M$ NaOH ($0.001\ M$ concentration).

This stock solution appeared to be stable for at least one month in the refrigerator. Preceding use, it was warmed to room temperature, then diluted 1 to 10 with distilled water, to make it $0.001\ M$ with respect to its NaOH concentration, and further with $0.001\ M$ NaOH to adjust its xanthine content to the required concentration. In order to avoid undue heating of the solutions during prolonged operations, both the sample and the reference solution were used only once in the analyses.

Measurements were made in the Beckman DU model spectrophotometer employing 1 cm. silica cells with the selector switch in the position 1.0. In the case of fine structure measurements readings were carried out at 1 m μ intervals throughout the entire spectral line; otherwise at 2 m μ intervals. The absorption spectra are presented in terms of per cent transmittance for practical purposes.

Results

The selection of an alkaline xanthine solution as an optical filtering agent for the spectrophotometric analyses of proteins initiated from the observations of Stimson and Reuter (10) who found that the single peak of the xanthine absorption curve with a maximum at 270 m μ at pH 3.0 and pH 7.0 was replaced at pH 11.0 by two peaks with maxima and minima at 240/228 and 278/258 m μ respectively.

Fig. 1 represents absorption curves of 2 \times 10⁻⁵, 4 \times 10⁻⁵, 6 \times 10⁻⁵, and 7 \times 10⁻⁵ M xanthine solutions at pH 11.0, expressed in % transmittance, using 0.001 M NaOH as the standard reference. Reference solutions of identical xanthine concentrations were employed as optical filters in the subsequent spectrophotometric measurements of protein spectra.

Fig. 2 illustrates the effect of a standard reference solution, containing an appropriate xanthine concentration, upon the absorption spectrum of egg albumin. Curve 1 shows the usual absorption curve of 0.1% egg albumin at pH 10.0, Curve 2 that of a 6 \times 10⁻⁵ M xanthine solution. The similarity of the two curves in the region at 250 to 300 m μ is obvious. According to previous observations (2, 3), this led us to suppose that reference solutions of analogous or similar xanthine contents may be well suited for a tentative spectrophotometric resolution of protein spectra. In agreement with these assumptions, Curve 3 indicates the absorption spectrum of the above egg albumin solution; at this time, however, the 6 \times 10⁻⁵ M xanthine solution was used as the standard reference, that is, as an optical filter.

For general information, it should be noted that in the subsequent figures, for comparative purposes, Curve 1 represents the absorption spectra of the respective proteins, using distilled water as the reference standard.

th a natic omos by h as culty ually

hrop

thich This ecule titunicanents

ution

DU was some ents,

were erum gent). cause

4 nimal

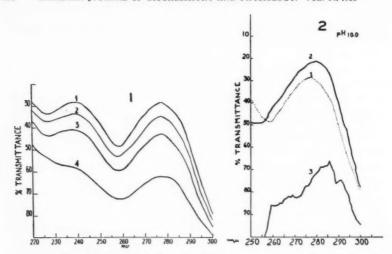


Fig. 1. Absorption spectra of various xanthine concentrations at pH 11.0. Standard reference solution 0.001 M NaOH. Curve 1. $2 \times 10^{-6} M$ xanthine.

Curve 3. $5 \times 10^{-6} M$ xanthine. Curve 4. $6 \times 10^{-6} M$ xanthine. Curve 2. $4 \times 10^{-6} M$ xanthine.

Fig. 2. Absorption spectrum of a 4×10^{-6} M xanthine solution (Curve 1) compared with that one of a 0.1% serum albumin solution at pH 7.3 (Curve 2) and its filtering effect on the spectrum of serum albumin when used as a reference standard (Curve 3).

Fig. 3, Curves 2 illustrate the absorption spectra of 0.1% bovine serum albumin employing $4 \times 10^{-5} M$ xanthine in the reference solution. It may be seen that the resolution of the spectra is more accentuated at pH 10.0 than at pH 7.3. In the first instance, shoulders and peaks are well recognizable and the spectrum shows an absorption maximum at 279 mµ. In the second case the spectrum reveals only a broad maximum at 278 mµ and is more similar in its features to that of Curve 1. Changing the concentration of the reference solutions to $6 \times 10^{-5} M$ xanthine, a sharpening of some of the peaks occurs at both pH values (Curves 3), while other peaks disappear, having been replaced by new ones. A well distinct new absorption maximum also becomes apparent at 285 m μ as a consequence of electronic transitions.

The absorption spectra of 0.1% egg albumin indicated in Fig. 4 differ considerably from those of serum albumin employing a reference solution of $4 \times 10^{-5} M$ xanthine. The spectra at either pH 7.3 or pH 10.0 show only one broad absorption maximum in the region at 280 mµ, though several subsidiary peaks are discernible. If the pH of the sample is at 10.0 and the xanthine concentration of the reference standard is increased to $6 \times 10^{-5} M$ the optical density of the spectrum decreases and a sharp absorption maximum at 280 m μ becomes visible followed by a second maximum at 291 m μ .

The spectra of 0.1% casein solutions at pH 7.3 and pH 10.0, using a standard reference solution of 4×10^{-5} , 6×10^{-5} , or 7×10^{-5} M xanthine, are presented

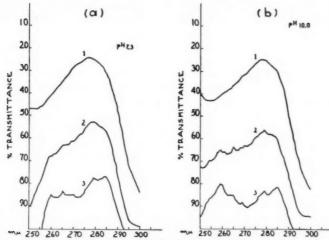


Fig. 3. The filtering effect of reference solutions containing two different xanthine concentrations on the absorption spectrum of serum albumin at pH 7.3 and pH 10.0 respectively.

d

d

um

be

nan ble

ond

ore

the

aks

also

con-

n of only

eral the

5 M

num

dard

nted

Curve 1. Samples: 0.1% serum albumin; reference solutions: distilled water of corresponding pH values.

Curve 3. Samples: 0.1% serum albumin; reference solutions: $4\times 10^{-6}~M$ xanthine. Curve 3. Samples: 0.1% serum albumin; reference solutions: $6\times 10^{-6}~M$ xanthine.

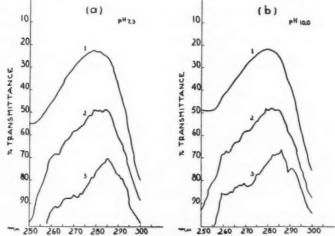


Fig. 4. The filtering effect of reference solutions containing two different xanthine concentrations on the absorption spectrum of egg albumin at pH 7.3 and pH 10.0 respectively.

respectively.

Curve 1. Samples: 0.1% egg albumin; reference solutions: distilled water of corresponding pH values.

Curve 2. Samples: 0.1% egg albumin; reference solutions: 4 × 10-8 M xanthine.

Curve 2. Samples: 0.1% egg albumin; reference solutions: $4\times 10^{-8}~M$ xanthine. Curve 3. Samples: 0.1% egg albumin; reference solutions: $6\times 10^{-8}~M$ xanthine.

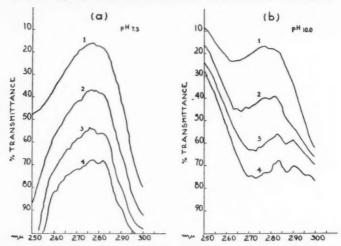


Fig. 5. The filtering effect of reference solutions containing two different xanthine concentrations on the absorption spectrum of casein at pH 7.3 and pH 10.0 respectively. Curve 1. Samples: 0.1% casein; reference solutions: distilled water of corresponding pH values.

Samples: 0.1% casein; reference solutions: 4 \times 10-5 M xanthine. Samples: 0.1% casein; reference solutions: 6 \times 10-5 M xanthine. Samples: 0.1% casein; reference solutions: 7 \times 10-5 M xanthine. Curve 2. Curve 3.

Curve 4.

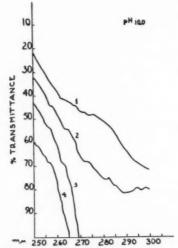


Fig. 6. The filtering effect of reference solutions containing three different xanthine concentrations on the absorption spectrum of gelatin at pH 10.0. Curve 1. Samples: 0.3% gelatin; reference solution: distilled water of a corresponding

pH value.

Curve 3. Samples: 0.3% gelatin; reference solution: $2\times 10^{-6}~M$ xanthine. Curve 3. Samples: 0.3% casein; reference solution: $4\times 10^{-6}~M$ xanthine. Curve 4. Samples: 0.3% gelatin; reference solution: $6\times 10^{-6}~M$ xanthine.

in Fig. 5. At pH 7.3 (Curves 2, 3, and 4) these spectra are rather uniform showing only one broad maximum at 280 m μ with only a few subsidiary peaks. At pH 10.0 the spectra reveal, however, a considerable alteration in comparison with the previous ones, according to the increased xanthine concentration of the reference solution (Curves 3 and 4). Thus for instance, two prominent maxima are present at 284 m μ and 290 m μ respectively, with a minimum between the two peaks at 287 m μ .

In Fig. 6 the absorption spectra of a 0.3% gelatin solution at pH 10.0 are given, using for the standard reference solution alternatively 4 \times 10⁻⁵, 6 \times 10⁻⁶, or 4 \times 10⁻⁷ M xanthine (Curves 2, 3, 4). In the absence of tyrosine and tryptophan the spectra are lacking in prominent features and only some peaks and shoulders are recognizable along the descending curves.

Discussion

The fine structure of the aromatic amino acids, phenyl alanine, tyrosine, and tryptophan is to some extent altered and shifted toward the longer wave lengths by their incorporation into the protein molecule. The sensitivity of the phenolic hydroxide in the tyrosine molecule to pH alterations may cause occasionally a further shift toward the longer wave lengths as was shown by Sizer and Peacock (9). On the other hand Crammer and Neuberger (5) found that the spectrum of ovalbumin did not change over the pH range 4.9 to 12.0. No appreciable shift could be observed in the present investigation in the spectra of egg albumin or serum albumin on, changing the pH of the sample from pH 7.3 to pH 10.0. However, an obvious sharpening in fine structure occurred indicating an increased intensity in electronic vibrations.

The observed long wave shift at pH 10.0 in casein spectra with the doubling of the absorption maximum in the region at 280 m μ presented in Curves 3 and 4 of Fig. 5 would indicate a specific configuration of the molecule.

The fine structure data of our own measurements at pH 10.0 are related in Table I to the wave lengths of vibrational fine structure bands in the spectra of serum albumin, ovalbumin, casein, and gelatin as determined by Beaven and Holiday (4, p. 319). Since the distribution of the absorption peaks and shoulders in the presented spectra depends on the xanthine concentration of the reference standard, similarities in the protein fine structure of both measurements become evident only if data of at least two xanthine concentrations are compared with those of the previous workers. In this way both measurements seem to be in good agreement with one another.

Conclusion

An increase in per cent transmittance with a consequent unveiling of the vibrational fine structures in the ultraviolet absorption spectra of some investigated proteins can be observed when a standard reference solution of an appropriate xanthine concentration is used for the spectrophotometric measurements. The data presented so far seem to compare well with the

ing

TABLE I

Ox serum albumin	Beaven and Holiday*		252.5	258.5		264.4	268.2	272.6	278.7	285.0	290.0
Bovine serum	Own	4 × 10-8 M	1	259		265	270	I	279	1	1
and	,,	$6 \times 10^{-8} M$ xanthine	1	259		262-265	269	1	279	285	1
Egg albumin	Beaven and Holiday		253.2	259.1	262.2	265.3	269.1	274.3	280.5	285.4	291.4
3	Own	$4 \times 10^{-5} M$	1	1	261	265	269	1	279-285	I	1
3	measurements ,	$6 \times 10^{-6} M$ xanthine	1	259	263	!	269	275	1	286	291.1
Casein	Beaven and Holiday		252.2	258.6		264.1	268.0	276.4		.283	291.0
25	Own	$4 \times 10^{-5} M$	1	1		264-265	590	277-280		282	
3	measurements	$6 \times 10^{-6} M$	1		1		1	1		283	290
Gelatin (sol.)	Beaven and Holiday		251.5	257.7	260.3	263.5	267.2	274.8		282.0	
3	Own	4 × 10-5 M	1	251-258	1	263-264	1	274		280-282	
3	measurements 6	$6 \times 10^{-4} M$ xanthine	1	1	Ţ	263	1	1		1	

* See (4, p. 332).

measurements of different workers, using more complicated methods. As present investigations have shown, the fine structure spectra obtained by this procedure proved to be particular to each protein examined so far, and consequently may serve for their spectrophotometric identification. If one considers also the gross shape of the respective protein spectra, individual differences become even sharper, revealing at the same time dissimilarities in their molecular configurations.

References

- 1. Annau, E. Can. J. Biochem. Physiol. 33: 826. 1955.
- 2. Annau, E. Can. J. Biochem. Physiol. 33: 833. 1955.
- 3. Annau, E. Can. J. Biochem. Physiol. 33: 1010. 1955.
- BEAVEN, G. H. and HOLIDAY, E. R. Ultraviolet absorption spectra of proteins and amino acids. In: Advances in protein chemistry. Vol. 7, p. 319. Academic Press Inc., New York. 1952.
- 5. CRAMMER, J. L. and NEUBERGER, A. Biochem. J. 37: 302. 1943.
- 6. HOLIDAY, E. R. J. Sci. Instr. 11: 166. 1950.
- 7. LAVIN, G. J. and NORTHROP, J. H. J. Am. Chem. Soc. 57: 874. 1935.
- 8. SINSHEIMER, R. L., SCOTT, J. F., and LOOFBOUROW, J. R. J. Biol. Chem. 187: 299. 1950.
- 9. Sizer, J. W. and Peacock, A. C. J. Biol. Chem. 171: 767. 1947.
- 10. STIMSON, M. M. and REUTER, M. A. J. Am. Chem. Soc. 65: 153. 1943.

THE GASTRIC ENDOCRINE AND EXOCRINE RESPONSE TO HISTAMINE IN DOGS AND EFFECT OF PASSAGE OF BLOOD THROUGH THE GASTRIC AND HEPATIC VESSELS ON ITS PEPSINOGEN CONTENT¹

By K. Kowalewski, S. T. Norvell, Jr., and Walter C. MacKenzie

Abstract

Subcutaneous histamine in dogs in a dose of 5 mgm. per kgm. of body weight provoked a significant increase in gastric pepsin secretion over a four-hour period. The increased pepsin production was accompanied by increased secretion of free HCl, increased volume of gastric juice, and elevation of plasma pepsinogen in the same experimental period. Plasma pepsinogen levels, before and after histamine, were investigated in a gastric artery, a gastric vein, the portal vein, a hepatic vein, and a cephalic vein. The enzyme concentration was significantly higher in the gastric vein than in the gastric artery and this difference was accentuated following histamine administration. These findings were attributed to endocrine secretion of pepsinogen by the zymogenic cells of the gastric mucosa. There was no evidence that pepsinogen concentration is altered as the blood traverses the liver.

Introduction

Previous studies in our laboratory have shown that uropepsin excretion and plasma pepsinogen concentration are significantly increased when histamine is administered to guinea pigs (10, 13), and the increase in plasma pepsinogen in dogs is proportionate to the dose of histamine given (12). Histamine is well established as a stimulant of gastric free acid, and in human subjects the secretion of hydrochloric acid has also been shown to be proportionate to the dose of histamine administered (1, 11).

Our present work is concerned with the simultaneous measurement of gastric endocrine and exocrine activity, in terms of plasma pepsinogen and gastric pepsin, as well as with the effect of the passage of blood through the stomach and liver on the pepsinogen content of its plasma. In these studies histamine has been employed to stimulate the production of plasma pepsinogen and of gastric pepsin.

That the output of gastric pepsin is paralleled by endocrine secretion of pepsinogen into the circulation has been previously postulated (7, 8). It was also demonstrated that under a variety of conditions the pepsinogen excreted by the kidneys, and presumedly derived from the plasma, is a constant fraction of the quantity of pepsinogen secreted into the stomach and converted into pepsin; this relationship has been referred to as the "endocrine–exocrine ratio" (8).

Histamine has not been widely used to stimulate gastric endocrine activity, perhaps, in part, because the status of histamine as a stimulant of gastric pepsin has been equivocal. Many authors have reported that histamine little affects or even diminishes the secretion of pepsin by the stomach (2, 3, 6).

¹Manuscript received August 22, 1955. Contribution from McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton, Alberta. On the other hand, Ivy and co-workers have demonstrated that histamine is an efficient stimulant of gastric pepsin secretion when an adequate dose is used (4, 5), and Stavraky has shown that under proper conditions histamine may cause the stomach to secrete pepsin (15). Because we had previously obtained good uropepsin and plasma pepsinogen responses to histamine, its effect on gastric pepsin seemed to warrant investigation.

Experimental

Mongrel dogs of both sexes, fed on commercial Miracle Dog Food and weighing from 8 to 27 kgm., were used in this experiment. All dogs were studied after a fasting period of at least 24 hr. and under sodium pentobarbital anesthesia (35 mgm. per kgm. body weight, intravenously). All dogs were protected against the systemic effects of histamine by promethazine HCl (Phenergan, Poulenc) given intramuscularly 30 min. before the subcutaneous injection of histamine dihydrochloride (Roche). The doses of antihistaminic and histamine were 5 mgm./kgm. body weight. It was previously demonstrated that neither pentobarbital anesthesia alone nor anesthesia and antihistaminic in combination in the doses employed results in a rise in plasma pepsinogen over a three-hour period in dogs (12).

Collection of Blood for Pepsinogen

In eight dogs plasma pepsinogen determinations were done on specimens obtained from a gastric vein, gastric artery, and cephalic vein. For canulation of the vessels each dog was subjected to laparotomy, and blood samples were taken immediately. One hour later the next sampling was performed and the antihistaminic was injected, followed in 30 min. by the injection of histamine. Posthistaminic samplings of blood were done one, two, three, and four hours after the injection of histamine.

In three dogs, histamine was given three hours before laparotomy. Immediately after the abdomen was opened, one blood sample was taken from each of four vessels: the portal vein, a hepatic vein, a gastric vein, and a gastric artery. This part of the experiment was done in order to compare the level of enzyme in various parts of the splanchnic vascular bed and to evaluate the possible influence of the liver on the concentration of plasma pepsinogen.

Collection of Gastric Juice

In eight dogs, gastric juice was aspirated at intervals and as completely as possible, through a Levin tube. The first aspiration was carried out at the time of the first blood sampling and the next aspiration was begun about 15 min. before histamine was injected and was completed as soon as histamine was administered. Posthistamine hourly outputs of gastric juice were collected in the same way, each aspiration being completed simultaneously with the blood sampling. Although this procedure does not guarantee the completeness of the aspiration of the hourly output, it seemed exact enough for the purpose of this study, and is essentially the same procedure used in most studies on gastric secretion.

stasma (12).

oroof and the

gen n of was eted tion

rine vity, stric ittle

into

, 6).

Satisfactory sampling of gastric content was obtained in six dogs. Results in two dogs were discarded, in one because it had undigested food in its stomach and in the other, the smallest of the series, because of difficulty in obtaining the samples.

Determination of Enzyme

Pepsinogen was determined according to the procedure of Mirsky et al. (14), except that plasma was digested with the substrate for 20 hr. instead of 24 hr. Pepsin was determined following the procedure of Anson as described by Aitken et al. (2), but in both procedures 2.5% solution of hemoglobin was used as a substrate. The results are expressed in terms of the amount of tyrosine released by the proteolytic action of 1 ml. of plasma or of the total hourly output of gastric juice.

Results

Plasma Pepsinogen

Table I presents the results of the study of plasma pepsinogen in samples collected from a gastric vein, a gastric artery, and the cephalic vein of eight dogs. Two blood samplings were taken before the injection of histamine in order to determine the effect of operative stress on the enzyme level. The average plasma pepsinogen value in the peripheral blood was 150 \pm 28 $\mu \mathrm{gm./ml.}$ immediately after cannulation and 146 \pm 20 $\mu \mathrm{gm./ml.}$ one hour later. The respective values for blood from gastric veins were 205 \pm 49 $\mu \mathrm{gm./ml.}$ and 213 \pm 42 $\mu \mathrm{gm./ml.}$ These results indicate that there is no significant difference between the two samplings.

The next four samplings were done at hourly intervals over a four-hour period after the administration of histamine. It may be seen in Table I that histamine induced a significant increase in plasma pepsinogen in all animals. Values of the plasma enzyme are increased in all samples, and in the majority of dogs the maximal rise occurred three to four hours after injection of histamine.

The coefficients of variation of values for pepsinogen in this group of animals were between 13.6 and 25.7% before the administration of histamine and between 11.5 and 26.3% after histamine.

An interesting finding was the notable difference in concentration of the plasma enzyme in the gastric vein and gastric artery. The ratio g.v.: g.a. (gastric vein: gastric artery) was from 1.20 to 1.27 before histamine and from 1.35 to 1.44 in the samples taken after histamine. The ratio g.a.: c.v. (gastric artery: cephalic vein) was 1.14 before histamine and from 1.07 to 1.09 after histamine. These results show that the concentration of plasma pepsinogen is highest in the blood from the gastric vein and progressively diminishes in the more distant vessels. This progressive change in the concentration of the enzyme is further illustrated in Table II.

In three dogs plasma pepsinogen was determined three and one-half hours after the injection of histamine and about 15 min. after laparotomy. Blood

TABLE I

ults its in

14), hr. by was t of otal

ples ight

e in The

28 nour 49

s no our that nals. ority ista-

mals and the g.a. from stric after ogen es in on of

ours Blood EFFECT OF HISTAMINE ON PLASMA PEPSINGGEN IN DOGS. SAMPLES OF BLOOD TAKEN FROM GASTRIC VEIN (G.V.), GASTRIC ARTERY (G.A.)

					Dog	00								Av.	Av. ratio
		-			-	9							J. Bond	0.0	0 3
Time of sampling	Origin	-	2	6	4	м	9		oc	Mea	Mean S.D.	3.	variation, "	तं तं	C.V.
Immediately after cannulation	N. V.	247	198	182	174	148	292	228	176	205	+	49	23.9	1.20	1
	18. ts.	189	160	156	140	140	246	190	152	171	+1	7	25.7		
	C.V.	153	122	125	137	135	200	182	148	150	+1	87	18.6		
One hour later	g.v.	258	194	177	167	235	273	224	176	213	+	42	7.61	1.27	1.14
	g.a.	175	125	133	137	182	258	171	156	167	+	42	25.1		
	c.v.	156	114	122	137	171	167	155	152	146	+1	20	13.6		
One hour after histamine	g.v.	809	650	459	418	413	418	425	400	474	+	86	20.6	1.40	1.00
	6.9	390	296	340	357	364	360	311	268	337	+1	43	12.7		
	c.v.	372	234	335	320	323	326	304	260	300	+1	43	13.9		
Two hours after histamine	R.V.	655	897	404	539	471	498	536	436	565	+1	149	26.3	1.41	1.07
	g.a.	406	520	418	300	342	420	403	288	300	+1	89	17.0		
	C.V.	380	475	387	357	312	413	380	280	37.3	+1	4.3	11.5		
Three bours after histamine	g.v.	782	852	968	573	46.3	500	492	520	598	+1	143	23.9	1.35	1.07
	g.a.	498	543	489	478	376	438	365	356	442		200	16.5		
	C.V.	459	539	463	387	345	420	353	340	413	± 52	7	12.5		
Four hours after histamine	g.v.	876	698	611	609	402	544	503	688	637	+ 10	091	25.1	1.44	1.09
	g.3.	573	809	06+	448	306	408	305	396	441		112	25.3		
	5	EAS	670	4.40	200	300	4 10 4		400			-			

TABLE II

Plasma pepsinogen (μ gm. per ml.) in samples of blood taken from Gastric vein (g.v.), portal vein (p.v.), hepatic vein (h.v.), and gastric artery (g.a.) of three dogs three and one-half hours after the injection of histamine

		Dog		
Sampling	9	10	11	Average
Gastric vein	604	544	482	543
Portal vein	422	380	352	384
Hepatic vein	372	352	320	348
Gastric artery	342	300	296	313
Ratio				
$\frac{g.v.}{p.v.}$	1.48	1.43	1.33	1.41
$\frac{p.v.}{h.v.}$	1.13	1.08	1.10	1.10
$\frac{h.v.}{g.a.}$	1.09	1.17	1.08	1.11
$\frac{g.v.}{g.a.}$	1.76	1.81	1.61	1.72

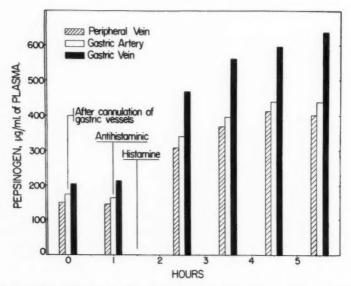


Fig. 1. Concentrations of plasma pepsinogen in various vessels before and after administration of histamine. Average for eight dogs.

was taken from a hepatic vein, the portal vein, a gastric vein, and a gastric artery. In this group of animals the ratio p.v.: h.v. was 1.10, the ratio h.v.: g.a. was 1.11, and the ratio g.v.: g.a. was 1.7. (Table II). These results indicate that passage of the blood through the liver has little influence on the level of pepsinogen. The difference between concentration of enzyme in the gastric vein and gastric artery is still more evident in these three dogs than in animals of the first group.

Fig. 1 presents graphically the concentrations of plasma pepsinogen found in various vessels.

Gastric Pepsin

v.).

Simultaneous sampling of gastric juice and blood for the study of pepsin and pepsinogen respectively was performed satisfactorily in six dogs. It is assumed that most of the gastric contents were evacuated by the procedure used in this experiment. The volume, the pH, the free hydrochloric acid, and the total pepsin were determined in each sample. The first collection was considered as the fasting content of the stomach, but as it was obtained shortly after laparotomy, the effect of operative stress on gastric secretion cannot be excluded.

TABLE III

STUDY OF GASTRIC CONTENT IN SIX DOGS BEFORE AND AFTER HISTAMINE

Volume expressed in ml., free HCl in meq./l., pepsin in mgm. of tyrosine per total collected gastric content

		Before h	istamine	I	lourly outputs	after histamine	
Dog	Gastric content	Fasting content	After 90 min.	1	2	3	4
1	Volume	24	28	50	109	80	40
	pH	1.2	1.8	1.3	0.9	1.0	1.0
	Free HCl	60	28	80	120	136	112
	Pepsin	38.8	30.4	125.0	245.8	184.0	92.0
2	Volume	10		20	130	110	30
	pH	2.7		1.6	0.8	0.8	0.8
	Free HCl	0		55	115	145	145
	Pepsin	16.2		106.9	275.9	236.0	97.6
3	Volume	30	31	50	106	100	81
	pH	1.9	3.1	1.5	1.5	0.9	0.8
	Free HCl	45	30	60	105	106	120
	Pepsin	42.0	36.0	100.0	208.0	196.8	124,0
4	Volume	32		40	90	95	68
	pH	1.2		1.1	0.8	0.8	0.8
	Free HCl	40		110	120	123	119
	Pepsin	34.8		116.0	128.0	126.0	103.0
5	Volume	25	9	42	120	110	60
	pH	2.0	3.8	1.3	1.0	0.9	0.9
	Free HCl	30	18	84	111	125	120
	Pepsin	32.5	10.3	159.5	250.5	240.0	107.5
7	Volume	19		82	122	110	20
	pH	3.0		1.0	0.8	0.8	0.8
	Free HCl	0		82	120	118	116
	Pepsin	21.7		138.5	233.0	204.5	122.3

fter

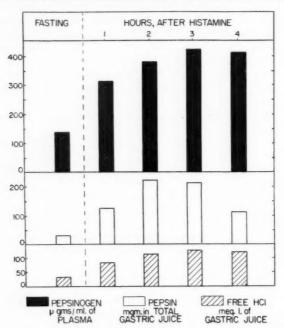


Fig. 2. Plasma pepsinogen of the peripheral blood correlated with gastric pepsin and free HCl. Average for six dogs.

The second collection of gastric juice was carried out 90 min. after the first and an adequate quantity of juice was obtained in only three dogs. The next four collections represent hourly outputs of gastric juice after the injection of histamine; the results are given in Table III. It is apparent from a review of this table that histamine affects significantly the volume, pH, free gastric acid, and the total output of pepsin. For all animals the maximal hourly output of pepsin occurs two hours after histamine, and there is no direct relationship between hourly pepsin outputs and the volumes or acidity of gastric juice.

The values of pepsin, free HCl, and plasma pepsinogen of the peripheral blood in the six dogs are presented together in Fig. 2.

Table IV gives a correlation of gastric pepsin output with plasma enzyme concentration found in the gastric veins. It can be concluded from Table IV that histamine provokes a significant increase in plasma pepsinogen and that the pepsinogen roughly parallels the rise in output in gastric pepsin; this parallelism is evident only during the first two hours after histamine, the plasma enzyme concentration then continuing to rise while the gastric enzyme output diminishes.

An attempt to correlate the hourly output of gastric pepsin with the values of plasma pepsinogen may be criticized for several reasons:

TABLE IV

VALUES OF GASTRIC PEPSIN CORRELATED WITH VALUES OF PLASMA PEPSINGEN CONCENTRATION IN GASTRIC VEINS IN SIX DOGS

Pepsin (fasting content and hourly output) expressed in mgm. per total collected gastric juice. Pepsinogen expressed in μ gm. per ml. of plasma

Dog	Determination	Fasting	Hours after histamine			
			1	2	3	4
1	Pepsin	38.8	125.0	245.8	184.0	92.0
	Pepsinogen	247	608	655	782	876
2	Pepsin	16.2	106.9	275.9	236.0	97.0
	Pepsinogen	198	650	897	852	869
3	Pepsin	42.0	100.0	208.0	196.8	124.0
	Pepsinogen	182	459	494	596	611
4	Pepsin	34.8	116.0	128.0	126.0	103.0
	Pepsinogen	174	418	539	573	609
5	Pepsin	32.5	159.5	250.5	240.0	107
	Pepsinogen	148	413	471	463	402
7	Pepsin Pepsinogen	21.7 228	138.5 425	233.0 536	204.5 492	122 503
	Pepsin Mean S.D. ± Coeff. of variation, %	32.0 10 31.2	124.5 22 17.6	223.5 48 21.4	197.9 42 21.2	107. 11 10.
	Pepsinogen Mean S.D. ± Coeff, of variation, %	196 36 18.3	495 105 21.2	568 163 28.7	626 157 25.0	645 190 29

 The value of plasma enzyme in a sample of blood gives information only about the concentration of pepsinogen at the moment of sampling.

2. The collected hourly output of gastric enzyme represents the work of the zymogenic cells during a given period and assumes complete evacuation of the gastric contents. The possibility of incomplete evacuation and mixing of non-evacuated gastric content with the newly secreted juice and of some loss of juice through the duodenum may influence the values obtained for gastric pepsin.

These factors should be considered in evaluating Table IV and Fig. 2.

Discussion

Histamine has long been considered to be a stimulant of the parietal cells of the stomach, and a direct relationship between the dose of histamine administered and the quantity of hydrochloric acid secreted has been demonstrated (1, 11). Histamine acts independently of the vagi and directly on the gastric mucosa (9) and many authors have identified endogenous histamine with gastrin (16).

The effect of histamine on the secretion of gastric pepsin, on the other hand, has been a subject of controversy (2, 3, 4, 5, 6, 15). It has been shown by others that the exocrine secretion of pepsin is paralleled by endocrine secretion of pepsinogen (7, 8) and the stimulant effect of histamine on plasma

The ion iew

the

d

tric irly ect of

eral

me IV hat

the

ues

and urinary pepsingen has been demonstrated in our laboratory. These findings permitted us to assume that histamine, given in adequate dosage, might stimulate the secretion of gastric pepsin; and our experiment, summarized in the present report, confirms this supposition. Histamine, administered as described in our experimental conditions, provoked a significant rise in plasma pepsinogen, in the hourly output of gastric pepsin, in the gastric free HCl, and in the volume of gastric juice.

Histamine stimulates both the endocrine and exocrine secretion of pepsino-Within the limitations of the experimental technique employed, a correlation between plasma pepsinogen and gastric pepsin is apparent after histamine administration.

The plasma pepsinogen concentration in blood from a gastric vein is greater than in that from other vessels, and this difference is accentuated following administration of histamine. Both of these findings are attributed to the secretion of pepsinogen into the blood by the zymogenic cells of the gastric mucosa.

Our data indicate that the liver does not alter the pepsinogen content of the blood that traverses it. The differences in portal vein and hepatic vein values are no greater than can reasonably be explained on the basis of dilution by blood from the hepatic artery.

Nothing is known about utilization or destruction of the enzyme elsewhere in the body, but it is widely accepted that plasma pepsinogen is excreted by the kidneys, probably in the glomerular filtrate, to form uropepsin or urinary pepsinogen.

Acknowledgments

The authors wish to thank Miss E. Schuetze for technical assistance. Histamine and Phenergan were kindly supplied respectively by Hoffman la Roche Co. and Poulenc Co. of Montreal.

References

- 1. ADAM, H. M., CARD, W. I., RIDDEL, M. J., ROBERTS, M., STRONG, J. A., and WOOLF, B. Brit. J. Pharmacol. 9: 323. 1954.
- AITKEN, M. A., SPRAY, S. H., and WALTERS, S. Clin. Sci. 13: 119. 1954.
 BABKIN, B. P. Secretory mechanism of the digestive glands. Paul B. Hoeber, Inc., New York. 1950.

- Hew Fork. 1930.

 4. BUCHER, G. R. and Ivy, A. C. Am. J. Physiol. 132: 654. 1941.

 5. BUCHER, G. R., Ivy, A. C., and Grey, J. S. Am. J. Physiol. 132: 698. 1941.

 6. GILMAN, A. and COWGILL, G. R. Am. J. Physiol. 97: 124. 1931.

 7. GRAY, S. J., RAMSAY, C. R., REIFENSTEIN, R. W., and BENSON, J. A. Gastroenterology, 25: 156. 1953. 1953.
- 8. JANOWITZ, H. D. and HOLLANDER, F. J. Appl. Physiol. 4: 53. 1951.
 9. KATZENELBOGEN, S., LOUCKS, R. B., and GANT, H. W. Am. J. Physiol. 128: 10. 1939.
 10. KOWALEWSKI, K. Can. J. Biochem. Physiol. 32: 553. 1954.
- 11. KOWALEWSKI, K., CONARD, V., and VAN GEERTRUYDEN, J. Compt. rend. soc. biol. 144:
- 29. 1950.
- 12. KOWALEWSKI, K. and NORVELL, S. T., JR. Can. J. Biochem. Physiol. 33: 599. 1955. 13. KOWALEWSKI, K., SCHNITKA, T. K., MOONEY, A. W., and HYDE, H. A. Acta gastroenterologica Belgica, 17: 832. 1954. 1954. 14. MIRSKY, A. T., FUTTERMAN, P., KAPLAN, S., and BROH-KAHN, R. H. J. Lab. Clin. Med.
- 40: 17. 1952.

 15. STAVRAKY, G. W. Federation Proc. 8: 151. 1949.

 16. TRACH, B., CODE, C. F., and WANGENSTEIN, D. H. Am. J. Physiol. 141: 78. 1944.

THE RELATIVE POTENCY OF ADRENAL CORTICOIDS BY THE THYMUS INVOLUTION METHOD¹

se

ge,

ared

in

ree

10a

ter

ter

ric

of

ein ion

ere

by

ary

nan

, B.

Inc.,

logy,

39.

144:

Med.

5. stroBy N. R. Stephenson

Abstract

An adrenal corticoid which has an α -ketolic grouping at C_{17} , a ketone or β -hydroxyl at C_{11} , a ketone at C_{24} , and a double bond in the 4,5 position is able to involute the thymus gland of the wearling rat. The thymolytic activity of an 11-oxycorticosteroid is increased approximately 3 to 3.5 times by the addition of an α -hydroxyl at C_{17} . The ability of a 17-hydroxycorticosteroid to cause thymic atrophy is enhanced 1.2 times by acetylation at C_{21} , 1.5 times by replacement of a ketone at C_{11} with a β -hydroxyl group, 4 to 5 times by the formation of a 1,2 double bond, and 8 to 10 times by the introduction of a fluorine atom in the α position at C_{2} . The potency of Δ 1-1-oxycorticosteroids relative to naturally occurring corticoids is significantly greater when the steroids are injected in an aqueous medium than when they are given in corn oil. The relative activities of adrenal corticoids as determined by the thymus involution method do not differ significantly from those obtained by other glucocorticoid bio-assays.

Introduction

It has been well established that chronic administration of 11-oxycorticosteroids will cause involution of the thymus gland (6, 10, 13, 14, 16, 19). In the intact weanling rat, a linear relationship with a negative slope was found to exist between the logarithm of the dose of adrenal corticoid and the weight of the thymus gland per 100 gm. of rat; this finding was employed to develop a bio-assay procedure for estimating the thymolytic activity of adrenal cortical extracts (16). The results obtained by this method did not differ significantly from those found by an assay based on the deposition of reducing substances in the liver of adrenalectomized male rats (15).

Santisteban and Dougherty (11), working with adrenalectomized mice, reported that a ketone or an hydroxyl group at C_{11} was essential for thymolytic activity. An hydroxyl group at C_{17} increased the activity, and maximum thymus atrophy was achieved when both C_{11} and C_{17} had hydroxyl groups. The relative potency of the adrenal corticoids was reported to be, in descending order: hydrocortisone, cortisone, corticosterone, and 11-dehydrocorticosterone.

The purpose of this paper is to determine quantitatively, in the intact weanling rat, the relative potency of various adrenal corticoids and to investigate the effect of changes in molecular configuration on the thymolytic activity. In addition, a study was made of the influence of the solvent used in the injection medium on the relative potency of 11-oxycorticosteroids.

Experimental

Weanling rats of the Wistar strain, 21–25 days old and weighing 35–45 gm., were used as the test animals. The assay procedure employed to determine the relative potency of the adrenal corticoids was similar to that described

¹Manuscript received November 14, 1955. Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.

previously (16) with the exception that the corticoids were injected subcutaneously three times daily, at 9 a.m., 1:30 p.m., and 5:00 p.m., for two days instead of twice daily for three days. The thymus glands were removed and weighed approximately 16 hr. after the last injection.

The low dose of the adrenal corticoid was adjusted to bring about a 25-35% involution of the thymus gland, while the high dose, equivalent to twice the low dose, usually produced a 50-60% involution. The low and high dose levels of hydrocortisone, used as the reference compound in most of the assays, were 5 and 10 mgm. per kgm. of rat respectively. The relative thymolytic activities of the adrenal cortical hormones were calculated on an equimolar basis.

The compounds under test were dissolved in either corn oil or an isotonic saline solution containing 10% ethyl alcohol, before being injected into the rats (16).

Results and Discussion

The Thymolytic Activity of the Adrenal Corticoids Relative to Hydrocortisone

The ability of various adrenal corticoids to involute the thymus gland of the intact wearling rat is demonstrated in Table I. Since both hydrocortisone and hydrocortisone acetate were used as reference compounds, a potency of 1.0 was arbitrarily assigned to them. The relative potencies of the corticoids as determined by the glycogen deposition (8) and the muscle work (7) tests are included in Table I for comparison with the thymus involution assay.

TABLE I RELATIVE POTENCY OF ADRENAL CORTICOIDS

	Thymus involution assay*			
Compound	Potency	Fiducial limits† $(P = 0.95)$	Glycogen deposition method	Muscle work test
Hydrocortisone	1.0		1.0†	1.08
11-Dehydrocorticosterone	0.23	0.21 - 0.26	0.32	0.25
Corticosterone	0.28	0.21 - 0.34	0.35	0.37
Cortisone	0.65	0.59-0.70	0.68	0.79
Prednisone	2.96	2.59-3.39		
Prednisolone	4.28	3.90-4.69		
Tetrahydrocortisone	0.0			
Adrenosterone	0.0			
11-Desoxycortisone (Reichstein's Compound S)	0.0			
Hydrocortisone acetate	1.0			
Cortisone acetate	0.76	0.67 - 0.84	1.01	
9α-Fluorohydrocortisone acetate	8.83	8.05-9.68	10.7 ± 2.3	
Desoxycorticosterone acetate	0.0		0.0	0.0

^{*}Corn oil was used as the solvent.

[†]Pahst, Sheppard, and Kuizenga (8). ‡Fried and Saho (5).

[§]Ingle and Kuisenga (7).

The values obtained by these three procedures show reasonably good agreement, indicating that each assay method is probably measuring the same type of "glucocorticoid" activity although the criterion of the response is quite different in each of the determinations.

l

e

e

e

e

n

C

e

of

ie

of

ls

ts

1.

Apparently the molecular configuration which is responsible for the atrophy of the thymus gland also stimulates gluconeogenesis in the liver and increases the ability of the muscle to do work. The data in Table I suggest that corticoids which are able to involute the thymus gland have a C₃ ketone accompanied by a double bond in the 4,5 position, an α -ketol grouping (-CH-CO-CH₂OH) at C₁₇, and either a ketone or a β-hydroxyl group at C₁₁. Desoxycorticosterone was inactive at a dose level of 65 mgm. per kgm. of rat, while desoxycortisone (Reichstein's Compound S) was ineffective at a dose of 30 mgm. per kgm. Neither of these corticoids has an oxygen atom at C_{11} . With adrenosterone, where the α -ketol side chain at C_{17} is replaced by a ketone, no thymolytic action was obtained at a dose level of 34 mgm. per kgm. of rat. The α,β unsaturated C₃-ketone is absent in tetrahydrocortisone and this C21-11-oxysteroid was found to be inactive at a dose of 40 mgm. per kgm. of rat. It is interesting to note that tetrahydrocortisone is formed in the liver by the enzymic reduction of cortisone (18) and has been reported to be the most abundant adrenocortical metabolite excreted by man (12).

In addition, Table I demonstrates that the introduction of a fluorine atom in the α position at C_9 brings about a 9- to 10-fold increase in both the thymolytic and the gluconeogenetic (5) activities. In a clinical experiment, Boland and Headley (2) found that 9α -fluorohydrocortisone possessed approximately 10 times the antiinflammatory activity of hydrocortisone in the treatment of rheumatoid arthritis. Although at the present time there is no adequate explanation for this marked enhancement of the biological activity, Todd and Hechter (17) have shown by experiments with rat liver brei and slices that the introduction of the 9α -fluorine atom decreased only slightly the normal rate of metabolism of the corticosteroid molecule.

	Thymus involution assay*	
Compound	Potency	Fiducial limits $(P = 0.95)$
Cortisone (Δ^4 -pregnene-17 α ,21-diol-3,11,20-trione) Hydrocortisone (Δ^4 -pregnene-11 β ,17 α ,21-triol-3,20-dione)	1.0 1.54	1.43-1.70
Prednisone ($\Delta^{1,4}$ -pregnadiene-17 α ,21-diol-3,11,20-trione) Prednisolone ($\Delta^{1,4}$ -pregnadiene-11 β ,17 α ,21-triol-3,20-dione)	1.0 1.37	1.24-1.52

^{*}Corn oil was used as the solvent.

The Relationship Between Molecular Structure and Thymolytic Activity

The results given in Table II indicate that replacement of a $C_{\rm II}$ -ketone by a $C_{\rm II}$ -hydroxyl raises the relative potency of a 17-hydroxycorticosteroid from 1.0 to approximately 1.5. Boland (1) obtained a result similar to this when he compared the antirheumatic potency of hydrocortisone with that of cortisone acetate. This was done by first stabilizing the patients on oral maintenance doses of cortisone acetate, transferring them to hydrocortisone, and then determining the amounts of the hormone preparations required to accomplish an equivalent degree of clinical improvement. The dosage ratio of hydrocortisone to cortisone acetate ranged from 1:1.4 to 1:1.7 with an average ratio of 1:1.59.

According to the data in Table III, the presence of an α -hydroxyl at C_{17} produces, on the average, a 3.5-fold increase in the thymolytic activity of an 11-oxycorticosteroid.

The relative potencies illustrated in Table IV reveal that the introduction of a double bond between C_1 and C_2 augments the thymus involuting action four to five times. A similar enhancement in activity was observed clinically (3, 4, 9) where the effectiveness of prednisone and prednisolone was compared with that of cortisone and hydrocortisone in the treatment of rheumatoid arthritis and selected dermatoses.

TABLE III $E {\tt FFECT} \ {\tt OF} \ {\tt AN} \ {\tt HYDROXYL} \ {\tt GROUP} \ {\tt AT} \ {\tt THE} \ C_{17} \ {\tt POSITION} \ {\tt ON} \ {\tt THE} \ {\tt THYMOLYTIC} \ {\tt ACTIVITY}$

	Thymus involution assay*	
Compound	Potency	Fiducial limits $(P = 0.95)$
11-Dehydrocorticosterone (Δ^4 -pregnene-21-ol-3,11,20-trione) Cortisone (Δ^4 -pregnene-17 α ,21-diol-3,11,20-trione)	1.0 3.48	2.83-4.54
Corticosterone (Δ^4 -pregnene-11 β ,21-diol-3,20-dione) Hydrocortisone (Δ^4 -pregnene-11 β ,17 α ,21-triol-3,20-dione)	1.0 3.57	2.90-4.76

^{*}Corn oil was used as the solvent.

 $\label{eq:table_iv} TABLE\ IV$ Effect of a double bond between C_1 and C_2 on the thymolytic activity

	Thymus involution assay*	
Compound	Potency	Fiducial limits $(P = 0.95)$
Cortisone (\triangle -pregnene-17 α ,21-diol-3,11,20-trione) Prednisone ($\Delta^{1,+}$ -pregnadiene-17 α ,21-diol-3,11,20-trione)	1.0	4.26-5.33
Hydrocortisone (Δ^4 -pregnene-11 β ,17 α ,21-triol-3,20-dione) Prednisolone (Δ^4 -pregnadiene-11 β ,17 α ,21-triol-3,20-dione)	1.0 4.28	3.90-4.69

^{*}Corn oil was used as the solvent.

 $\label{eq:table V} \textbf{TABLE V}$ Effect of $C_{z1}\text{-ester}$ formation on the thymolytic activity

	Thymus involution assay*	
Compound	Potency	Fiducial limits $(P = 0.95)$
Hydrocortisone Hydrocortisone acetate Hydrocortisone t-butylacetate	1.0 1.18 0.78	1.09-1.27 0.60-0.92
Cortisone Cortisone acetate	1.0 1.21	1.09-1.34

^{*}Corn oil was used as the solvent.

bу

om en of ral ne, to tio an

an

on

on

lly ed oid

its

TABLE VI

Effect of the solvent in the injection medium on the relative potency of the adrenal conticoids

	Corn oil		10% ethyl alcohol in saline	
Compound	Thymolytic activity	Fiducial limits $(P = 0.95)$	Thymolytic activity	Fiducial limits $(P = 0.95)$
Hydrocortisone	1.0		1.0	
Cortisone	0.65	0.59-0.70	0.59	0.45 - 0.77
Prednisone	2.96	2.59-3.39	4.12*	3.28-5.68
Prednisolone	4.28	3.90-5.23	5.40*	4.72-6.19
Cortisone	1.0		1.0	
Prednisone	4.74	4.26-5.33	7.44*	5.75-11.33

^{*}Increase in thymolytic activity significant at P = 0.05.

Acetylation at C_{21} also influences the relative potency of the adrenal corticoids. The values shown in Table V indicate that the acetate esters of both cortisone and hydrocortisone are 1.2 times more active than the free alcohols. On the other hand, the C_{21} -t-butylacetate ester of hydrocortisone was found to be approximately 20% less active than the free alcohol. This change in the thymolytic activity probably depends upon the alteration in both the solubility and the availability of the corticoid after esterification at the C_{21} -hydroxyl group.

Effect of the Injection Medium on the Relative Potency of the Adrenal Corticoids

Previously the thymus involuting action of hydrocortisone was shown to be significantly greater when it was administered in vegetable oil than when it was given in an aqueous medium (16). Table VI demonstrates the relative thymolytic activities of 11-oxycorticosteroids following injection in either corn oil or an aqueous solution containing 0.9% sodium chloride and 10% ethyl alcohol. The potency of hydrocortisone relative to cortisone, and that of

prednisolone relative to prednisone, was unaffected by the injection medium. However, the involuting activity of prednisone and prednisolone relative to hydrocortisone was significantly higher when the corticoids were given in an aqueous solution. A similar significant increase in the relative potency was observed when prednisone and cortisone were compared. Since the dose of prednisone or prednisolone was only about 25% of that of cortisone or hydrocortisone, the enhancement of the relative potency observed when 10% ethanol in saline was used as the solvent may be related to a more efficient absorption of the Δ^1 -11-oxycorticosteroid from the site of injection. This finding clearly indicates that it is necessary to specify the type of injection medium employed when comparing the thymolytic action of compounds like prednisone with that of the naturally occurring adrenal corticoids.

Acknowledgment

The adrenal corticosteroids used in this investigation were generously provided by Merck and Co. Ltd., Montreal (cortisone, hydrocortisone acetate, hydrocortisone t-butylacetate, 11-dehydrocorticosterone, corticosterone, 11desoxycortisone (Reichstein's Compound S), 9α -fluorohydrocortisone, prednisone, and prednisolone) and Schering Corporation, Bloomfield, New Jersey (hydrocortisone, 11-dehydrocorticosterone, adrenosterone, tetrahydrocortisone, prednisone, and prednisolone). The author wishes to thank Mr. A. J. Bayne for his technical assistance.

References

- 1. BOLAND, E. W. Merck Rept. 62: 12. 1953.
- 2. BOLAND, E. W. and HEADLEY, N. E. Ann. Rheumatic Diseases, 13: 291. 1954.
- 3. Bunim, J. J., Pechet, M. M., and Bollet, A. J. J. Am. Med. Assoc. 157: 311. 1955. 4. DEMARTINI, F., BOOTS, R. H., SNYDER, A. I., SANDSON, J., and RAGAN, C. J. Am. Med.
- 1955. Assoc. 158: 1505. 5. FRIED, J. and SABO, E. F. J. Am. Chem. Soc. 76: 1455. 1954.
- Ingle, D. J. Proc. Soc. Exptl. Biol. Med. 44: 174. 1940.
- 7. INGLE, D. J. and KUIZENGA, M. H. Endocrinology, 36: 218. 1945.
- 8. Pabst, M. L. R., Sheppard, R., and Kuizenga, M. H. Endocrinology, 41: 55. 1947.
- 9. Robinson, H. M. J. Am. Med. Assoc. 158: 473. 1955.
- 10. SANTISTEBAN, G. A. Anat. Record, 115: 366. 1953.
- 11. Santisteban, G. A. and Dougherty, T. F. Endocrinology, 54: 130. 1954.
- Schneider, J. J., Lewbart, M. L., Levitan, P., and Lieberman, S. J. Am. Chem. Soc. 77: 4184. 1955.
- SELYE, H. Brit. J. Exptl. Pathol. 17: 234. 1936.
 SELYE, H. Am. J. Physiol. 116: 141. 1936.

- STEPHENSON, N. R. Can. J. Biochem. Physiol. 32: 218. 1954.
 STEPHENSON, N. R. Can. J. Biochem. Physiol. 32: 689. 1954.
- 17. Todd, D. and Hechter, O. Arch. Biochem. and Biophys. 56: 268. 1955.
- 18. TOMKINS, G. and ISSELBACHER, K. J. J. Am. Chem. Soc. 76: 3100. 1954.
- 19. Wells, B. B. and Kendall, E. C. Proc. Staff Meetings Mayo Clinic, 15: 133. 1940.

LIPID AND WATER LEVELS IN THE KIDNEYS OF ALBINO RATS BEARING WALKER CARCINOMA 2561

ım.

to:

an was

e of Iro-

0%

ent 'his

ion

like

ısly

ate,

11-

red-

sev

orti-

. J.

55.

7.

Soc.

10

Med.

By Eldon M. Boyd and Arne O. Tikkala

Abstract

The kidneys of 54 pairs of twin albino rats, one inoculated and one not inoculated with Walker carcinoma 256, were analyzed for water, total lipid, neutral fat, total fatty acids, total cholesterol, ester cholesterol, free cholesterol, and phospholipid, calculated as gm. per 100 gm. nonlipid dry weight. Compared with the kidneys of their nontumor-bearing littermates, the kidneys of tumor-bearing rats exhibited no significant change in wet weight and in concentration of total lipid, neutral fat, total fatty acids, and ester cholesterol, free was a significant increase in concentration of water, total cholesterol, free cholesterol, and phospholipid. The increase was toward concentrations of corresponding elements in Walker carcinoma 256. The changes became evident in animals bearing tumors weighing 20 to 40% or more of host weight.

Introduction

Analysis of aliquots of Walker carcinoma 256 has revealed that the tumor maintains, throughout its growth in the albino rat, relatively high concentrations of water, phospholipid, total cholesterol, and free cholesterol, and relatively low concentrations of neutral fat per unit dry weight (8). The carcass of littermate, nontumor-bearing albino rats has what may be described as an opposite type of composition, that is, relatively high concentrations of neutral fat and relatively low concentrations of the other components (5). As Walker carcinoma 256 grows, the concentration of water, phospholipid, total cholesterol, and free cholesterol increases and the concentration of neutral fat decreases in the carcass of the host rat (4, 16).

The changes in concentration have been categorized collectively as the hydrolipotropic shift toward tumor levels (6). Certain organs and tissues of the host rat develop, during growth of Walker carcinoma 256, a similar hydrolipotropic shift toward tumor levels (4, 7, 9, 10, 14). The changes which comprise the hydrolipotropic shift are not gross changes demonstrable in single animals. They can be proved to occur by analysis of differences in a sufficiently large group of twin animals, one twin grafted and the other twin not grafted with Walker carcinoma 256.

The investigation described in this report was concerned with the water and lipid content of the kidneys of albino rats bearing Walker carcinoma 256. The concentrations per unit nonlipid dry weight exhibited a hydrolipotropic shift which became evident in the kidneys of rats bearing what might be described as tumors of intermediate size.

¹Manuscript received in original form July 20, 1955, and as revised, December 13, 1955. Contribution from the Department of Pharmacology, Queen's University, Kingston, Ontario. This investigation was supported by a grant from the National Cancer Institute of Canada. The authors wish to acknowledge the cooperation of Elizabeth A. Bentley, Carl E. Boyd, Elinor M. Crandell, Valmore Fontaine, J. Gilbert Hill, H. Douglas McEwen, and Monica E. Murdock. A report of this investigation was presented at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics, University of Iowa, Iowa City, Iowa, September, 1955.

Method

The lipid and water contents of the kidneys were determined upon 54 littermate pairs of albino rats of Wistar strain, one of each littermate pair inoculated and one not inoculated with Walker carcinoma 256. The technique has been described (5, 8). The albino rats used have been inbred in the animal quarters of the Department of Pharmacology at Queen's University since 1937. The animals were fed Purina fox chow checkers and water ad libitum. A summary of data upon age and weight of body and tumor is presented in Table I. Tumors were removed after the rats were sacrificed with chloroform at the end of 14 to 42 days of tumor growth. The wet weight of the excised tumor is expressed as a percentage of host weight. Distribution in the size of tumors in the rats studied is indicated in Fig. 1. Statistical methods used throughout this report are those of Croxton (11). Thirty-four of the littermate pairs were males and 20 littermate pairs were females. Both sexes were included because of possible sex differences in the kidney (13), but none was found in the present study, except a lower renal wet weight in the smaller female rat.

Both kidneys were removed from the tumor-bearing rat and its nontumorbearing littermate. The wet weight of the kidneys was not significantly affected by growth of Walker carcinoma 256, but the kidneys comprised a significantly lower proportion of body weight and a significantly higher proportion of host weight in the tumor-bearing littermate (Table II). This applied to both males and females.

TABLE I

THE AGE AND WEIGHT OF 54 ALBINO RATS BEARING WALKER CARCINOMA 256 AND OF 54 NONTUMOR-BEARING LITTERMATES, WITH CORRESPONDING DATA UPON THE TUMOR

Measurement	Tumor-bearing albino rats, mean ± S.D.	Nontumor-bearing littermates, mean ± S.D.
Body weight at inoculation—gm.	125 ± 39	123 ± 39
Age of tumor—days	24 ± 6	mercan mercan
Final body weight—gm.	228 ± 69	197 ± 49
Final age of rat—days	68 ± 10	68 ± 10
Weight of tumor—gm.	70 ± 50	
Weight of tumor host-gm.	158 + 39	-
Weight of tumor as of host weight	45 ± 28	

TABLE 11
THE WEIGHT OF THE KIDNEYS IN ALBINO RATS BEARING WALKER CARCINOMA 256

Measurement of renal weight	Tumor-bearing rats, mean ± S.D.	Nontumor-bearing littermates, mean ± S.D.	C_0 change in tumor- bearing rats significant at $P = 0.01$ or less
Grams wet weight	1.76 ± 0.38	1.82 ± 0.37	None
c total body weight	0.809 ± 0.134	0.932 ± 0.099	-13.2
host weight	1.16 ± 0.187	0.932 ± 0.099	+24.2

An aliquot of the kidneys was removed for determination of water content. A further aliquot was weighed, ground with cleaned sand, and extracted with alcohol-ether for determination of lipid composition by an adaptation of the oxidative micromethods of Bloor as noted by Boyd *et al.* (10).

Results

A summary of data upon the concentration of each measured renal constituent in the control, nontumor-bearing albino rats, calculated as gm. per 100 gm. of nonlipid dry weight, is given in Table III. The lipid concentrations are slightly higher than those based upon calculation as gm. per 100 gm. total dry weight, as reviewed by Bloor (3) and by Deuel (12). The figures given in column two of Table III may be viewed as ratios of gm. of the measured components to 100 gm. of nonlipid dry weight.

The value of each measurement upon a nontumor-bearing albino rat was subtracted from the corresponding value in its tumor-bearing twin to obtain a difference. Calculations of differences were possible upon 48 of the 54 pairs of twins, insufficient data being available upon the other six pairs. The mean difference, in gm. per 100 gm. nonlipid dry weight, was then calculated as a percentage of the mean concentration of the component under study, in gm. per 100 gm. nonlipid dry weight, in the nontumor-bearing twins. These mean percentage changes in concentration have been listed in column three of Table III. A t test was applied to the probability of the differences being zero and the determined P values are given in Table III.

There were found to be significant increases averaging 14 to 17 per cent in the levels of total cholesterol, free cholesterol, phospholipid, and water in the kidneys of tumor-bearing albino rats. There were no significant shifts in the mean levels of total lipid, neutral fat, total fatty acids, and ester cholesterol. When differences in concentration between individual pairs of twins were

TABLE III

LIPID AND WATER LEVELS IN THE KIDNEYS OF TWIN ALBINO RATS, ONE BEARING AND ONE NOT BEARING WALKER CARCINOMA 256

Measurement	Nontumor-bearing twins: mean ± S.D.*	Tumor-bearing twins: mean difference from nontumor-bearing twins†	P value of mean difference
Total lipid	14.11 ± 4.33	+ 8.5	0.1
Neutral fat	4.36 ± 3.33	-6.0	0.7
Total fatty acids	9.62 ± 3.61	+ 5.9	0.4
Total cholesterol	1.54 ± 0.46	+14.2	0.001
Ester cholesterol	0.20 ± 0.42	+16.4	0.7
Free cholesterol	1.35 ± 0.26	+13.7	< 0.001
Phospholipid	8.26 ± 2.59	+16.0	< 0.001
Water	390 ± 30	+17.3	< 0.001

^{*}Grams per 100 gm. nonlipid dry weight. †Per cent of mean in nontumor-bearing twins.

gher This

54

pair

que the

sitv

ad

r is

iced

ight

tion

tical

four

Both

but

the

nor-

ntly

ed a

ing

morficant less plotted against the weight of the tumor, as per cent of host weight, in the tumor-bearing twin, there was found to be no significant shift in concentration of total lipid, neutral fat, total fatty acids, or ester cholesterol at any period during the growth of Walker carcinoma 256.

n

The calculated values for neutral fat and ester cholesterol inherit the errors in four and two direct measurements respectively. Hence the values for neutral fat and ester cholesterol are more variable than are those of the other measurements, as may be seen from the standard deviations given in column two of Table III. These analytical variations may or may not have contributed to the inability to prove significant shifts in concentration of neutral

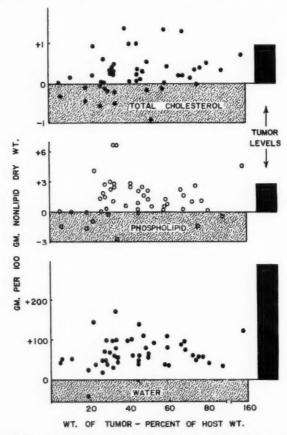


Fig. 1. Shifts in the concentration of total cholesterol, phospholipid, and water in the kidneys of albino rats bearing Walker carcinoma 256. (The results are plotted as the value in each tumor-bearing twin minus the value in its nontumor-bearing twin. The solid columns to the right indicate the mean level in Walker carcinoma 256 (10) minus the mean level in the kidneys of nontumor-bearing albino rats.)

he

on

od

ors

for

ner

mn

tri-

ral

he

he

fat and ester cholesterol in the kidneys of albino rats bearing Walker carcinoma 256. The term neutral fat is applied to residual fatty acids calculated to be not present in phospholipid and cholesterol ester fractions, and estimated as triglyceride fat. The neutral fat fraction may contain free fatty acids or other saponifiable combinations of fatty acids or other substances with corresponding solubilities.

The relationships of increases in concentration of total cholesterol, phospholipid, and water to increase in tumor mass have been illustrated in Fig. 1. In this figure have been plotted the differences between the tumor-bearing and nontumor-bearing littermates of each of the 48 sets of twins. It may be seen that the increases in concentration appeared in albino rats bearing tumors which weighed 20 to 40% of host weight. These increases were maintained in animals bearing larger tumors.

The concentration of total cholesterol, phospholipid, and water was significantly less in the kidneys of nontumor-bearing albino rats than in the peripheral actively growing part of Walker carcinoma 256 (10). The mean concentration in the kidneys was subtracted from the mean concentration in Walker carcinoma 256 and the differences have been shown as solid columns to the right of Fig. 1. This illustrates that the shift in concentration of these three components was toward the level in the tumor.

The concentration of free cholesterol in the kidneys of nontumor-bearing albino rats was significantly lower than the concentration in Walker carcinoma 256 (10). The concentration of free cholesterol in the kidneys of tumor-bearing albino rats increased at about the same point as did concentration of total cholesterol, shown in Fig. 1, toward the level in Walker carcinoma 256.

Discussion

The data described indicate that there occurred in the kidneys of albino rats bearing Walker carcinoma 256, a hydrolipotropic shift toward tumor levels. Shifts toward tumor levels were possible in the instances of total cholesterol, free cholesterol, phospholipid, and water levels and all four shifts were present. The concentration of neutral fat in the kidneys of nontumorbearing albino rats was not significantly different from that in Walker carcinoma 256 (10). Hydrolipotropic shifts in neutral fat levels were not possible, theoretically. Actually, the concentration of renal neutral fat did not shift in the tumor-bearing albino rats. Aoki (1) reported an increase in the concentration of total cholesterol, free cholesterol, and water, and a decrease in the concentration of phospholipid and fatty acids per unit wet weight of kidneys in hepatoma-bearing rats.

It must be remembered that description of these hydrolipotropic shifts applies to *concentrations* of water and lipids per unit nonlipid dry weight. The increases in concentration noted above and in Table III could not have followed automatically upon the mean decrease in concentration of neutral fat shown in Table III, since no lipid was included in the reference denominator, nonlipid dry weight. Increases in the ratio of weight of phospholipid,

Method

The lipid and water contents of the kidneys were determined upon 54 littermate pairs of albino rats of Wistar strain, one of each littermate pair inoculated and one not inoculated with Walker carcinoma 256. The technique has been described (5, 8). The albino rats used have been inbred in the animal quarters of the Department of Pharmacology at Queen's University since 1937. The animals were fed Purina fox chow checkers and water ad libitum. A summary of data upon age and weight of body and tumor is presented in Table I. Tumors were removed after the rats were sacrificed with chloroform at the end of 14 to 42 days of tumor growth. The wet weight of the excised tumor is expressed as a percentage of host weight. Distribution in the size of tumors in the rats studied is indicated in Fig. 1. Statistical methods used throughout this report are those of Croxton (11). Thirty-four of the littermate pairs were males and 20 littermate pairs were females. Both sexes were included because of possible sex differences in the kidney (13), but none was found in the present study, except a lower renal wet weight in the smaller female rat.

Both kidneys were removed from the tumor-bearing rat and its nontumorbearing littermate. The wet weight of the kidneys was not significantly affected by growth of Walker carcinoma 256, but the kidneys comprised a significantly lower proportion of body weight and a significantly higher proportion of host weight in the tumor-bearing littermate (Table II). This applied to both males and females.

TABLE I

The age and weight of 54 albino rats bearing Walker carcinoma 256 and of 54 nontumor-bearing littermates, with corresponding data upon the tumor

Measurement	Tumor-bearing albino rats, mean ± S.D.	Nontumor-bearing littermates, mean ± S.D.
Body weight at inoculation—gm.	125 ± 39	123 ± 39
Age of tumor—days	24 ± 6	
Final body weight—gm.	228 ± 69	197 ± 49
Final age of rat—days	68 ± 10	68 ± 10
Weight of tumor-gm.	70 ± 50	
Weight of tumor host-gm.	158 ± 39	
Weight of tumor as % of host weight	45 ± 28	

TABLE II

THE WEIGHT OF THE KIDNEYS IN ALBINO RATS BEARING WALKER CARCINOMA 256

Measurement of renal weight	Tumor-bearing rats, mean ± S.D.	Nontumor-bearing littermates, mean ± S.D.	% change in tumor- bearing rats significant at $P = 0.01$ or less
Grams wet weight total body weight	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	1.82 ± 0.37 0.932 ± 0.099	None -13.2
host weight	1.16 ± 0.187	0.932 ± 0.099	+24.2

An aliquot of the kidneys was removed for determination of water content. A further aliquot was weighed, ground with cleaned sand, and extracted with alcohol-ether for determination of lipid composition by an adaptation of the oxidative micromethods of Bloor as noted by Boyd *et al.* (10).

54

air

que the

ity

ad

ris

ced

ght

ion

ical

our

oth

but

the

or-

itly

d a

her

his

ıg

orcant

Results

A summary of data upon the concentration of each measured renal constituent in the control, nontumor-bearing albino rats, calculated as gm. per 100 gm. of nonlipid dry weight, is given in Table III. The lipid concentrations are slightly higher than those based upon calculation as gm. per 100 gm. total dry weight, as reviewed by Bloor (3) and by Deuel (12). The figures given in column two of Table III may be viewed as ratios of gm. of the measured components to 100 gm. of nonlipid dry weight.

The value of each measurement upon a nontumor-bearing albino rat was subtracted from the corresponding value in its tumor-bearing twin to obtain a difference. Calculations of differences were possible upon 48 of the 54 pairs of twins, insufficient data being available upon the other six pairs. The mean difference, in gm. per 100 gm. nonlipid dry weight, was then calculated as a percentage of the mean concentration of the component under study, in gm. per 100 gm. nonlipid dry weight, in the nontumor-bearing twins. These mean percentage changes in concentration have been listed in column three of Table III. A t test was applied to the probability of the differences being zero and the determined P values are given in Table III.

There were found to be significant increases averaging 14 to 17 per cent in the levels of total cholesterol, free cholesterol, phospholipid, and water in the kidneys of tumor-bearing albino rats. There were no significant shifts in the mean levels of total lipid, neutral fat, total fatty acids, and ester cholesterol. When differences in concentration between individual pairs of twins were

TABLE III

LIPID AND WATER LEVELS IN THE KIDNEYS OF TWIN ALBINO RATS, ONE BEARING AND ONE NOT BEARING WALKER CARCINOMA 256

Measurement	Nontumor-bearing twins: mean ± S.D.*	Tumor-bearing twins: mean difference from nontumor-bearing twins†	P value of mean difference
Total lipid	14.11 ± 4.33	+ 8.5	0.1
Neutral fat	4.36 + 3.33	- 6.0	0.7
Total fatty acids	9.62 ± 3.61	+ 5.9	0.4
Total cholesterol	1.54 ± 0.46	+14.2	0.001
Ester cholesterol	0.20 ± 0.42	+16.4	0.7
Free cholesterol	1.35 ± 0.26	+13.7	< 0.001
Phospholipid	8.26 ± 2.59	+16.0	< 0.001
Water	390 ± 30	+17.3	< 0.001

^{*}Grams per 100 gm. nonlipid dry weight. †Per cent of mean in nontumor-bearing twins.

plotted against the weight of the tumor, as per cent of host weight, in the tumor-bearing twin, there was found to be no significant shift in concentration of total lipid, neutral fat, total fatty acids, or ester cholesterol at any period during the growth of Walker carcinoma 256.

The calculated values for neutral fat and ester cholesterol inherit the errors in four and two direct measurements respectively. Hence the values for neutral fat and ester cholesterol are more variable than are those of the other measurements, as may be seen from the standard deviations given in column two of Table III. These analytical variations may or may not have contributed to the inability to prove significant shifts in concentration of neutral

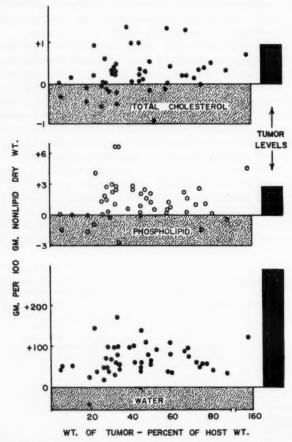


Fig. 1. Shifts in the concentration of total cholesterol, phospholipid, and water in the kidneys of albino rats bearing Walker carcinoma 256. (The results are plotted as the value in each tumor-bearing twin minus the value in its nontumor-bearing twin. The solid columns to the right indicate the mean level in Walker carcinoma 256 (10) minus the mean level in the kidneys of nontumor-bearing albino rats.)

the

ion

iod

ors

for

her

mn

tri-

tral

he

he

he

fat and ester cholesterol in the kidneys of albino rats bearing Walker carcinoma 256. The term neutral fat is applied to residual fatty acids calculated to be not present in phospholipid and cholesterol ester fractions, and estimated as triglyceride fat. The neutral fat fraction may contain free fatty acids or other saponifiable combinations of fatty acids or other substances with corresponding solubilities.

The relationships of increases in concentration of total cholesterol, phospholipid, and water to increase in tumor mass have been illustrated in Fig. 1. In this figure have been plotted the differences between the tumor-bearing and nontumor-bearing littermates of each of the 48 sets of twins. It may be seen that the increases in concentration appeared in albino rats bearing tumors which weighed 20 to 40% of host weight. These increases were maintained in animals bearing larger tumors.

The concentration of total cholesterol, phospholipid, and water was significantly less in the kidneys of nontumor-bearing albino rats than in the peripheral actively growing part of Walker carcinoma 256 (10). The mean concentration in the kidneys was subtracted from the mean concentration in Walker carcinoma 256 and the differences have been shown as solid columns to the right of Fig. 1. This illustrates that the shift in concentration of these three components was toward the level in the tumor.

The concentration of free cholesterol in the kidneys of nontumor-bearing albino rats was significantly lower than the concentration in Walker carcinoma 256 (10). The concentration of free cholesterol in the kidneys of tumor-bearing albino rats increased at about the same point as did concentration of total cholesterol, shown in Fig. 1, toward the level in Walker carcinoma 256.

Discussion

The data described indicate that there occurred in the kidneys of albino rats bearing Walker carcinoma 256, a hydrolipotropic shift toward tumor levels. Shifts toward tumor levels were possible in the instances of total cholesterol, free cholesterol, phospholipid, and water levels and all four shifts were present. The concentration of neutral fat in the kidneys of nontumorbearing albino rats was not significantly different from that in Walker carcinoma 256 (10). Hydrolipotropic shifts in neutral fat levels were not possible, theoretically. Actually, the concentration of renal neutral fat did not shift in the tumor-bearing albino rats. Aoki (1) reported an increase in the concentration of total cholesterol, free cholesterol, and water, and a decrease in the concentration of phospholipid and fatty acids per unit wet weight of kidneys in hepatoma-bearing rats.

It must be remembered that description of these hydrolipotropic shifts applies to *concentrations* of water and lipids per unit nonlipid dry weight. The increases in concentration noted above and in Table III could not have followed automatically upon the mean decrease in concentration of neutral fat shown in Table III, since no lipid was included in the reference denominator, nonlipid dry weight. Increases in the ratio of weight of phospholipid,

total cholesterol, free cholesterol, and water to weight of nonlipid dry weight may be related to shifts in the nitrogen content of the latter fraction (17). They indicate a direct or indirect effect of Walker carcinoma 256.

The total amount of renal water, dry weight, and all of the lipids except ester cholesterol and phospholipid was significantly less in the tumor-bearing albino rats when total amounts were calculated as mgm. per kidneys per kgm. total (tumor + host) body weight. This may be taken to signify that there were fewer of these renal elements to accommodate the needs of the tumor and host. On the other hand, when total renal amounts were calculated as mgm. per kidneys per kgm. host weight (minus tumor), there were significant increases in the total amounts of all renal elements measured except neutral fat. This might be taken to indicate that the kidneys were in a relatively better position to accommodate the needs of the host alone. Whatever be the significance of these differences, it is apparent that the loss of host wet weight in tumor-bearing albino rats (Table I) is not accompanied by any significant loss in renal wet weight (Table II). No loss of renal wet weight had been noted previously in rats bearing Flexner-Jobling carcinoma (15) and Sarcoma R1 (2).

Finally, it is of value to note the relationship of the hydrolipotropic shifts to age of Walker carcinoma 256 as used for correlation by some authors such as Mider (17). By correlating shifts in concentration with age of tumor, it was found that the significant shifts appeared after the tumor had reached an age of three to four weeks.

References

Aoki, C. Gann, 32: 100. 1938.
 Babson, A. L. Cancer Research, 14: 89. 1954.
 Bloor, W. R. Biochemistry of the fatty acids and their compounds, the lipids. Reinhold Publishing Corporation, New York. 1943.
 Boyd, E. M., Boyd, C. E., Hill, J. G., and Rayinsky, E. Can. J. Biochem. Physiol. 32: 359. 1954.
 Boyd, E. M., Connell, M. L., and McEwen, H. D. Can. J. Med. Sci. 30: 471. 1952.
 Boyd, E. M., Fontaine, V., and Hill, J. G. Proc. Can. Physiol. Soc. 16. 1954.
 Boyd, E. M., Fontaine, V., and Hill, J. G. Can. J. Biochem. Physiol. 33: 69. 1955.
 Boyd, E. M. and McEwen, H. D. Can. J. Med. Sci. 30: 163. 1952.
 Boyd, E. M., McEwen, H. D., and Murdoch, M. E. J. Natl. Cancer Inst. 1956. In press.

press.

10. Boyd, E. M., McEwen, H. D., and Shanas, M. N. Can. J. Med. Sci. 31: 493. 1953.

11. Croxton, F. E. Elementary statistics with applications in medicine. Prentice-Hall, Inc., New York. 1953.

12. Deuel, H. J. The lipids their chemistry and biochemistry, Volume 11: Biochemistry. Interscience Publishers, Inc., New York. 1955.

13. Gardner, W. U. In Advances in cancer research. Vol. 1. Edited by J. P. Greenstein and A. Haddow. Academic Press, Inc., New York. 1953. p. 173.

14. Hill, J. G., Fontaine, V., and Boyd, E. M. J. Pharmacol. Exptl. Therap 113: 28. 1955.

14. HILL, J. 1955.

LePage, G. A., Potter, V. R., Busch, H., Heidelberger, C., and Hurlbert, R. B. Cancer Research, 12: 153. 1952.
 McEwen, H. D. In Proceedings of the first Canadian cancer research conference. Vol. 1. Edited by R. W. Begg. Academic Press, Inc., New York. 1955. p. 141.
 Mider, G. B. In Proceedings of the first Canadian cancer research conference. Vol. 1. Edited by R. W. Beggard Academic Press. Inc. New York 1955. p. 141.

Edited by R. W. Begg. Academic Press, Inc., New York. 1955. p. 120.

THE DEGRADATION OF DESOXYRIBONUCLEIC ACID DURING ALKALINE HYDROLYSIS¹

ght 17).

ept

per hat

the

ted nifi-

ept

n a

ost any

ght

and

ifts

uch

r, it

lan

Rein-

. 32:

952.

55.

În

Hall,

stry.

stein

: 28.

R. B.

ol. 1.

ol. 1.

By R. O. HURST

Abstract

The extent of alkaline degradation of desoxyribonucleic acid (DNA) was determined by following the formation of organic phosphate soluble in 10% trichloroacetic acid solution and the liberation of ammonia. Hydrolysis of DNA with 1 N sodium hydroxide at 100% C, for four hours was required to effect complete formation of acid-soluble oligonucleotides and the ammonia liberated was equivalent to the estimated amino nitrogen content of the DNA.

Introduction

The difference in alkaline lability between desoxyribonucleic acid (DNA) and ribonucleic acid (RNA) has been attributed by Brown and Todd (6) to the possibility of formation of intermediate cyclic phospho-triester linkages in the RNA molecule because of the presence of an unsubstituted hydroxyl group in the pentose component. The occurrence of a desoxy carbon in the DNA pentose precludes the formation of this type of phospho-anhydride group and this accordingly is considered to explain the relative stability of DNA to mild treatment with alkali whereas RNA can be readily hydrolyzed to mononucleotides.

Tipson (15) has credited Bredereck and Jochman (4) with the accomplishment of hydrolyzing DNA to oligonucleotides by means of dilute sodium hydroxide solution at 60° C. This was actually claimed by Bredereck and Müller (5) who used a sample of DNA prepared previously by Bredereck and Caro (3) according to the method of Levene (9). This isolation procedure causes the depolymerization of DNA to oligonucleotides as indicated by electrometric titration studies (10). Bredereck and Müller (5) warmed their crude preparation of DNA to 60° C. in the presence of about 0.1 N sodium hydroxide in order to effect the precipitation of ferric hydroxide and purified their material by an alcohol - hydrochloric acid extraction procedure. Bredereck and Jochman (4) correctly considered that the isolation of a pentabasic DNA was due to alkaline degradation but they did not discuss the conditions required to effect the formation of oligonucleotides from highly polymerized DNA. It was therefore apparent that this problem should be reinvestigated in order to define properly the nature of the alkaline degradation products of DNA and the conditions required to produce oligonucleotides.

We therefore began the study of the alkaline degradation of DNA using the acid-soluble phosphorus method of Little and Butler (11) as a basis for following the reaction.

¹Manuscript received October 20, 1955. Contribution from the Department of Biochemistry, Queen's University, Kingston, Ontario.

Materials and Methods

Desoxyribonucleic Acid

This was prepared from calf thymus glands by the method of Marko and Butler (12). The preparation used in this work had the following properties: nitrogen-phosphorus ratio, 1.69; specific viscosity per gm. phosphorus per liter, 66; extinction per mole of phosphorus per liter (in water) at 2600 Å, 8720, and at 2300 Å, 3380.

Phosphorus Determinations

Phosphorus determinations were made by the method of Beveridge and Johnson (2).

Acid-soluble phosphorus determinations were based on the solubility of the hydrolysis products of DNA in 10% trichloroacetic acid solution according to the procedure used by Little and Butler (11) for following the activity of desoxyribonuclease.

Determination of Free Ammonia

The aeration procedure of Sobel, Mayer, and Gottfried (14) was modified and used in conjunction with the colorimetric method of Russel (13) for the determination of ammonia nitrogen. Preliminary experiments showed that direct determinations using the procedure of Russel (13) on solutions of adenine, guanine, cytosine, or DNA not treated with alkali gave values equivalent to a blank determination and therefore this procedure would not give incorrect results because of deamination by the alkaline phenol reagent.

For the determination of a standard curve a stock solution of ammonium sulphate containing 4.00 μ gm. of nitrogen per ml. was used and samples of 0.50 to 3.00 ml. were placed in 10-ml. volumetric flasks, which were kept in an ice bath during the addition of the reagents. Two milliliters of 0.01 N sulphuric acid solution, then 2.0 ml. of phenol reagent, 1.0 ml. of hypochlorite reagent, and one drop of 0.001 M manganous sulphate were added to each. The phenol and hypochlorite reagents were prepared according to the method of Russel (13). Additions to a series of flasks were all carried out concurrently with the precaution that the stoppers were replaced immediately after the additions. Finally the solutions were diluted to volume with water, stoppered tightly, and mixed by inversion. After mixing, the stoppers were loosened and the flasks placed in a boiling water bath for five minutes and then cooled. The flasks were then inverted several times in order to ensure the completion of color development and the optical density was read at 6250 Å within one hour on a Beckman model DU quartz spectrophotometer.

In the aeration technique a series of 50 ml. conical centrifuge tubes were connected according to the procedure described by Sobel *et al.* (14). Since boric acid interfered in the colorimetric estimation it was decided to trap the ammonia in 4.00 ml. of a 0.01 N sulphuric acid solution, which was found to be adequate. After aeration was completed a 2.00 ml. sample of this solution was then used for determination of ammonia nitrogen as previously described.

If it were necessary to dilute the sample this was done with 0.01 N sulphuric acid solution. Because of the sensitivity of the method of Russel to small variations in pH, it was necessary to standardize the addition of sulphuric acid solution for all determinations. The use of 2.0 ml. of the alkaline phenol reagent was found to be sufficient to counteract the 0.02 meq. of sulphuric acid present.

In our procedure an equal volume of saturated potassium carbonate solution was added to effect the liberation of ammonia from ammonium sulphate standard samples and, in order to ensure complete recovery of the liberated ammonia, an aeration time of two hours was employed. A good recovery with the aeration method outlined above could be obtained as shown in Table I. The graph of these results showed a linear relationship.

TABLE I

Comparison of the direct method for the determination of ammonia nitrogen with the indirect aeration method

	Optical density at 6250 Å			
Ammonium sulphate – standard, µgm. of nitrogen	Non-aerated	Aerated for 2 hr. into 0.01 N H ₂ SO ₄		
2	0.148	0.158		
4	0.221	0.235		
6	0.304	0.305		
8	0.376	0.372		
10	0.440	0.443		
12	0.507	0.515		

Alkaline Hydrolysis of Desoxyribonucleic Acid

d

S:

ıd

of ng

of

ed ne at of es

m of

in N

0-

to

to

ed li-

th

rs

es to

at

re

ce

he

to

on ed.

Several experiments were carried out to determine the relative lability of DNA at different temperatures and concentrations of alkali. Ten or 20 ml. of a 0.5% solution of DNA containing the appropriate equivalents of alkali was placed in a 50 ml. conical centrifuge tube fitted with a reflux condenser and a lead to a trap containing 4.00 ml. of 0.01 N sulphuric acid solution. The rubber tube connections were closed by clamps and the solution was heated in a water bath. When the period of hydrolysis was completed, ammonia-free air was passed through to remove any free ammonia present in the system and then the reflux condenser was removed and an equal volume of saturated potassium carbonate solution was added to the hydrolyzate. The aeration assembly as described by Sobel et al. (14) was attached immediately and the system aerated for two hours into the same sulphuric acid solution. If serious frothing occurred it was sufficient to add a few drops of octyl alcohol to the hydrolyzate. Ammonia determinations were then made on samples of the 0.01 N sulphuric acid solution. This procedure was particularly advantageous for determination of small amounts of ammonia since a fivefold concentration of the sample is obtained by aerating from a 20.0 ml. hydrolyzate into 4.00 ml. of the 0.01 N sulphuric acid solution.

In general these experiments were carried out with paired samples, one of which was used for the aeration procedure and the other for determination of acid-soluble phosphorus after neutralization of the alkaline digest. Aeration of the alkaline solution after addition of potassium carbonate to half saturation was not considered to cause additional liberation of ammonia since unhydrolyzed nucleic acid showed no deamination under the conditions employed (Table II).

TABLE II

EFFECT OF CONCENTRATION OF ALKALI, TEMPERATURE, AND DURATION OF HYDROLYSIS ON THE DEGRADATION OF DNA

Time, hr.	Temperature, °C.	Concentration of alkali	Acid-soluble phosphorus as Conference phosphate	Ammonia, M per 4 M. of nitrogenous base
24	60	0.25 N NaOH 4.06 N K ₂ CO ₃	11.8	0.02
48	60	4.00 N K2CO3	16.2	0.02
96	60	46	31.1	0.45
144	60	44	39.5	0.71
1	100	0.1 N NaOH	4.4	0.01
1	100	1.0 N NaOH	75.1	2.05
2	100	66	80.3	2.23
3	100	46	92.0	2.65
4	100	44	100.0	3.00

Discussion

The results of alkaline hydrolysis of DNA at 60° C. shown in Table II indicate that this treatment does not give complete formation of acid-soluble oligonucleotides even in six days and therefore the observations of Bredereck and Müller (5) can only be interpreted as due to the use of degraded DNA. Helleiner and Butler (7) have also found that DNA is not readily hydrolyzed by hot alkali.

On the basis of the composition of DNA from calf thymus (average of four determinations corrected for 100% recovery) reported by Hurst, Marko, and Butler (8) there are 2.88 moles of amino nitrogen per 4 moles of nitrogenous base. If all of the ammonia obtained in treating DNA with 1 N sodium hydroxide at 100° C. for four hours is derived from the amino groups of adenine, guanine, and cytosine it is apparent from the data presented in Table II that this would indicate complete deamination of DNA under these conditions.

Beale, Harris, and Roe (1) have criticized the use of concentrations of alkali greater than 0.3 N at 37° C. for analysis of the purine-pyrimidine composition of RNA because of the deamination of cytosine to uracil, but no one has reported any serious errors introduced in these methods due to

deamination of adenine and guanine. It is significant in this regard that treatment of DNA with 0.1 N sodium hydroxide at 100° C, for one hour produced negligible amounts of ammonia and acid-soluble phosphorus.

Little and Butler (11) have reported the presence of an unknown constituent of DNA, possibly protein in nature, in their preparations and it is therefore possible that some other component may be contributing to the formation of ammonia or that the purine and pyrimidine bases may undergo more extensive degradation than deamination.

The extreme conditions required to obtain acid-soluble oligonucleotides give a further indication of the difference between RNA and DNA with respect to the mechanism for alkaline hydrolysis of the internucleotide phosphate ester linkages.

Acknowledgment

The author wishes to acknowledge the technical assistance given by Mr. John Frei. This work was supported by a grant from the National Research Council of Canada.

References

- 1. BEALE, R. N., HARRIS, R. J. C., and ROE, E. M. F. J. Chem. Soc. 1034. 1952.
- 2. Beveridge, J. M. R. and Johnson, S. E. Can. J. Research, E, 27: 159. 1949.
- 3. Bredereck, H. and Caro, G. Z. physiol. Chem. 253: 170. 1938.
- 4. Bredereck, H. and Jochman, I. Ber. deut. chem. Ges. 75: 395. 1942. 5. Bredereck, H. and Muller, G. Ber. deut. chem. Ges. 72: 115. 1939.
- 6. Brown, D. M. and Todd, A. R. J. Chem. Soc. 52. 1952.
- 7. HELLEINER, C. W. and BUTLER, G. C. Can. J. Chem. 33: 705. 1955.
- 8. HURST, R. O., MARKO, A. M., and BUTLER, G. C. J. Biol. Chem. 204: 847. 1953.
- 9. LEVENE, P. A. J. Biol. Chem. 53: 441. 1922.
- 10. LEVENE, P. A. and SIMMS, H. S. J. Biol. Chem. 65: 519. 1925.
- LITTLE, J. A. and BUTLER, G. C. J. Biol. Chem. 188: 695. 1951.
 MARKO, A. M. and BUTLER, G. C. J. Biol. Chem. 190: 165. 1951.
- 13. Russel, J. A. J. Biol. Chem. 156: 457. 1944.

1 S n f n e

f

t 0

- 14. Sobel, A. E., Mayer, A. M., and Gottfried, S. P. J. Biol. Chem. 156: 355. 1944.
- 15. Tipson, R. S. Advances in Carbohydrate Chem. 1: 245. 1946.

ELECTROPHORESIS OF AUTOPROTHROMBIN AND BIOTHROMBIN¹

By Walter H. Seegers, Takeshi Abe,2 and Richard L. Fenichel

Abstract

Concentrates of biothrombin and autoprothrombin were prepared from purified prothrombin and subjected to electrophoresis. The mobility of biothrombin is less than that of prothrombin. The mobility of autoprothrombin is less than that of biothrombin. The biothrombin and autoprothrombin preparations examined were not electrophoretically homogeneous. Prothrombin dissociates during electrophoresis at high voltage gradients. Since this does not occur with biothrombin and autoprothrombin, it seems likely that electrophoretically homogeneous derivatives of prothrombin can be obtained with further purification work.

Introduction

Biothrombin preparations are obtained from purified prothrombin by interactions with Ac-globulin, purified platelet factor 3, Linadryl, and calcium ions (2). Biothrombin is the naturally occurring thrombin and is quite different from citrate thrombin which is obtained by the autocatalytic activation of purified prothrombin in 25% sodium citrate solution. Although citrate thrombin has been studied by electrophoresis no suitable preparations of biothrombin have been available for that purpose. Autoprothrombin, another derivative of prothrombin prepared in this laboratory, has only recently become available in adequate quantities for study (2). The latter is obtained by interacting purified prothrombin with calcium ions, a small amount of Ac-globulin, and purified platelet factor 3.

Materials and Methods

Bovine biothrombin and autoprothrombin were prepared by methods described by Seegers and Alkjaersig (2). These methods of preparation yield products which are still mixed with the ingredients of the reaction mixture required to derive them from prothrombin. However, the degree of purity achieved makes these products most useful for studies which were hitherto not possible. Electrophoresis experiments were performed with veronal buffer pH 8.6, ionic strength 0.1, at a voltage gradient of 7.5, according to a standard procedure previously described (1). The Aminco–Stern apparatus was used.

Results

Since biothrombin and autoprothrombin are derived from prothrombin, it was logical to include the latter in our study. In some of the earliest work, its mobility was found to be nearly identical with plasma albumin (3). Its

¹Manuscript received December 20, 1955.

Contribution from the Department of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Michigan. This investigation was supported by a research grant H-1467C from the National Heart Institute, National Institutes of Health, Public Health Service.

²Home address: Department of Medicine, University of Tokyo, Toyko, Japan.

mobility was also reported to be 6.7×10^{-5} cm.²/v./sec. (ascending boundary) (4), and even though efforts at further purification did not yield a more active product, there was invariably some material of lower mobility. In the ultracentrifuge a single component is found. The quantity of the second component varied from one preparation to another seldom exceeding 10% of the total protein, and probably represented decomposition of the prothrombin. We have now increased the voltage gradient from 5.4 to 7.5 and find that the ascending boundary pattern of prothrombin breaks into two major components at the end of two hours of electrophoresis. This break does not occur with the descending boundary pattern, or even with the ascending boundary pattern at the end of one hour. We believe that the prothrombin dissociates during the electrophoresis and that this is more likely to occur with the higher voltage gradient.

Biothrombin and autoprothrombin do not show a split into two large components on either the ascending or descending boundary pattern. Thus these derivatives of prothrombin appear to be more stable than the parent protein (Fig. 1). They do not dissociate at a relatively high voltage gradient. This result corresponds with other indications of comparatively greater stability of autoprothrombin and biothrombin reported by Seegers and

Alkjaersig (2).

d

is

h

IS

1.

1.

ll

ds on on ee

re th d-

n,

ts

ity

antice.

Table I summarizes our data on mobility. The mobility of biothrombin is slower than that of prothrombin, and that of autoprothrombin is less than that of biothrombin. Associated with the preparation of autoprothrombin there was a component possessing the electrophoretic properties of prothrom-

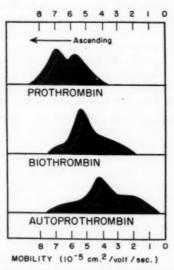


Fig. 1. Electrophoretic patterns in veronal buffer at 120 min., pH $8\cdot6$, ionic strength $0\cdot1$, and voltage gradient $7\cdot5$.

TABLE I

ELECTROPHORETIC MOBILITY OF AUTOPROTHROMBIN, PROTHROMBIN, AND BIOTHROMBIN

Material	u ×	u × 10 ⁵		Time u × 10 ⁵		C ~
Material	Asc.	Desc.	- min.	Asc.	min.	Composition, %
Prothrombin Fast peak Slow peak	6.4	6.4	, 60	7·4 6·2	120 120	49 51
Autoprothrombin†	4.3	4.0	60	4.7	120	
Biothrombin Major peak Fast peak Slow peak	6.5	5.6	60	5·8 7·3 4·7	110 110 110	78 12 10

*No separation in 60 min.

†After 150 min. of electrophoresis the autoprothrombin separated into a major peak representing 87% of the total area and a peak representing 13% of the total protein. The mobility of the former was $4\cdot 8\times 10^{-6}$ cm.²/v./sec. while that of the latter was $6\cdot 1\times 10^{-6}$ cm.²/v./sec.

bin. Another point of interest relates to the fact that autoprothrombin has the same mobility as citrate autoprothrombin. The latter was studied by Seegers, McClaughry, and Fahey (4) who recognized an intermediate that forms long before thrombin activity is detected in the autocatalytic activation of prothrombin with 25% sodium citrate solution.

The electrophoresis experiments indicate that the purification of biothrombin and autoprothrombin has advanced to such an extent that it should be possible to remove the remaining impurities and obtain these substances as single components. In contrast with prothrombin, they are relatively stable.

References

- 1. REINER, M., FENICHEL, R. L., and STERN, K. G. Acta Haematol. 3: 202. 1950.
- 2. SEEGERS, W. H. and ALKJAERSIG, N. Arch. Biochem. and Biophys. In press.
- 3. SEEGERS, W. H., LOOMIS, E. C., and VANDENBELT, J. M. Proc. Soc. Exptl. Biol. Med. 56:70. 1944.
- 4. SEEGERS, W. H., McClaughry, R. E., and Fahey, J. L. Blood, 5:421. 1950.

COMPARATIVE BIOCHEMICAL STUDIES ON NORMAL AND ON POLIOMYELITIS INFECTED TISSUE CULTURES

I. OBSERVATIONS ON SYNTHETIC NUTRIENT MIXTURES INCUBATED WITH TISSUE CULTURES OF NORMAL KIDNEY¹

By Ernest Kovacs

Abstract

Illustrative examples are given of change in turbidity values of complete and incomplete media used for in vitro cultivation of rhesus monkey kidney tissue. Pools of routine material were examined. The main interest was focused on enzymes directly or indirectly connected with nucleic acid metabolism. The presence and behavior of acid and alkaline monoesterases, 5-nucleotidase, simple nucleotidases, two different types of ribonuclease, and two desoxyribonucleases are described and activity of other enzymes occasionally demonstrated. As a working hypothesis, the bearing of these findings on cell physiology and on pathology of poliomyelitis virus infection is discussed.

Introduction

IS

y

at

i-

1-

e

18

ly

d.

Since the original work of Carrel (4) on the cultivation of animal tissues outside the organism, intensive studies have been made on the standardization of these techniques (39). The most important theoretical and technical contribution was the preparation of synthetic media, introduced by White (48), and further developed by Fischer (11) and especially by Morgan, Morton, and Parker (37). The observations to be described have been carried out on the latter type of media in intimate contact with surviving or growing tissue fragments or cells. We feel that for the more successful cultivation of viruses in vitro an amplified knowledge of the biology and biochemistry of cultured cells is an elementary necessity. Basic information, applying relatively simple techniques, can be obtained from the observations on the nutrient during tissue cultivation. Some constituents have already been examined. For instance inorganic phosphates have been investigated by Brues et al. (2) in complete and incomplete media. Glucose utilization in synthetic nutrient mixtures was studied by Franklin and associates (14, 15). Other substances have been demonstrated in culture fluids, usually connected with glycolysis (49). More information is available for the cultivated tissues, using various methods (20, 2, 22, 38, 45, 49). The substances present in used media are known in general as "metabolites" or "toxic substances" (18), but more qualitative and quantitative data are necessary to interpret the changes occurring in chemically defined media during the preinfective phase of virus production (8, 5, 9, 41, 50, 51). We have followed the most important procedures in this field by biochemical means. The demonstration of various enzyme systems acting in these fluids is first described as the main part of the present paper. Other publications of this cycle deal with studies in normal

¹Manuscript received August 8, 1955.
Contribution from the Department of Hygiene and Preventive Medicine, University of Toronto, Toronto, Ontario. This work was financed by a public health research grant.

tissue cultures (31) and in normal kidney (32), both homogenized in similar nutrients, in order to arrive at a better understanding of the fate of tissues infected with various strains of poliomyelitis virus (33).

Materials and Methods

The assay material was derived from two sources. Flask culture fluids as used for his unpublished virus assays were obtained through the courtesy of Dr. A. E. Franklin. Roller-tube tissue cultures were kindly supplied by Dr. D. R. E. MacLeod and by Dr. G. H. Macmorine. Both were routine material for virus titration experiments. Minced kidney fragments of about 1 mm. in size were used for the former, suspended in various media in 25-ml. Erlenmeyer flasks to give about one per cent wet weight concentration, and incubated at 37° C. for five days. Then the fluid was decanted and the product of three parallel experiments pooled and sent to our laboratory where the used nutrient, termed contact medium (CM), was examined. The nutrient fluids used for the flask cultures2 were usually balanced salt solutions, such as Tyrode's or Earle's mixture, alone or supplemented by glucose and/or by various amino acids. Synthetic nutrient mixtures 697 and 635 of Healy and associates (20) were also assayed, in their original form or combined with some new ingredients. All media contained phenol red (15 mgm. per liter) as indicator, and antibiotics. After the routine five days' incubation the CM was replaced by fresh nutrient and the tissue culture inoculated with polio virus.

Roller-tube tissue cultures were prepared by the well known techniques used in the Connaught Medical Research Laboratories (9, 50) and abroad (41) with the synthetic medium 199 of Morgan et al. (37) to which 0.5% horse serum was added. The pH was adjusted by addition of NaHCO₃, in 0.19% final concentration. The chopped and pooled rhesus kidney cortex was treated with trypsin and trypsinized cells suspended with the synthetic media plus adjuvants in ratio of 1:1400 to 1600. Two milliliters of this original cell suspension was explanted in Pyrex test tubes (10 × 150 mm.) closed with rubber stoppers, and incubated in roller drums at 37° C. for six days, as a rule. Then the fluid was changed, substituted by a double volume (4 ml.) of 199 without horse serum and 0.19% NaHCO3 (final concentration). This "wash fluid" bathed the tissues in the incubator usually for 16 to 24 hr. to free the explant from horse serum. A second fluid change was made after this washing, and consisted of fresh 2 ml. of 199 plus 0.19% bicarbonate (final concentration). The tissues incubated with this third fluid were ready for inoculation with poliomyelitis virus. Since August 1955 in the Connaught Laboratories a modified form of 199 has been used, called medium 597, which does not contain pyrimidines, purines, and pentoses.3

The following contact media were examined: the first fluid after few hours' contact and after six days' incubation; washing media (one, three, and seven days old); the third fluid after one day (eight day-old tissue culture) and after

²Unpublished personal communications of Dr. A. E. Franklin, Connaught Medical Research Laboratories.

³Recommended by Dr. R. C. Parker and introduced by G. H. Macmorine.

six days (when the explant was 14 days old). To exclude sampling variations it was decided to pool at least 100 tubes from each batch in our laboratory and to use pools of several hundred tubes from the production department where large quantities were prepared and the fluid replaced routinely. The check on these tissue cultures included inspection of the color of the indicator and direct microscopic observation of the tissues. Counting of the cells was made occasionally in the assay department, and the original 1:1600 dilution was found to contain about 400,000 cells per ml. More emphasis is given to the work on roller-tube tissue cultures where the initial dilution is known, a more complete medium (37) is employed, and one is dealing practically with one cell type.

To obtain some information about the amount of material present, and in that way assure the connection between this work and the assays on tissue culture (31) or on surviving tissue homogenates (32), turbidity tests were made (35). This rough means was checked by Kjeldahl-nitrogen (46), non-protein nitrogen (26), colorimetric protein (1) determinations, and by ultraviolet spectrophotometry (27) in preliminary assays. It was found that about 70–75% of the turbidity index is due to proteins. Because of the large amount of assay material the studies on proteins, nucleic acid (NA), carbohydrates, and phosphates will be reported separately (34).

Studies were made on fresh kidney cortex (1:9) and on acetone-dried kidney powder (1:19) by blending the cortex and powder with water and with various synthetic media for 3 to 10 min.; these blends were then centrifuged for 20 min. and the extraction controlled by turbidimetry. Mean experimental

error was \pm 2.5%.

ar

es

ds

SI.

pi.

ne

ut

nl.

nd

he

ere

ent

as

pi.

nd

ith

as

as

sed

11)

rse

ted

lus

cell

ith

ıle.

99

ish

the

ng,

n).

ith

s a

ain

irs

ven

ter

arch

Ultraviolet spectrophotometry was carried out, as previously described (27), with a Beckman Model DU instrument. Samples were diluted with distilled water, or with phosphate buffers of the desired pH, and read against water, buffer, or against similarly diluted media.

Various standard techniques served for the demonstration of enzymes. Phosphatases were measured by the techniques of Shinowara et al. (43, 30). Nucleases were assayed by methods previously reported (28, 29, 36, 44). 5-Nucleotidase was measured by the methods of Reis (40) and of others (17, 21) adapted to our purpose (30). Simple nucleotidases (47) were estimated as previously by the use of an equivalent mixture of four nucleotides of pentose nucleic acid (30). Nucleosidases were detected by differential spectrophotometry of Kalckar (23). Xanthine oxidase was demonstrated by Kalckar's spectrophotometric procedure (23). For choline esterase a spectrophotometric technique was adopted (24). Phosphate determination was carried out by Fiske and SubbaRow's technique for inorganic phosphates, by King's method for total acid soluble phosphorus. In preliminary work it was observed that some fluids of suspended flask cultures contained a large amount of eluted material; therefore they were homogenized by microgrinders of the Potter-Elvehjem type. In recorded experiments, however, completely clear contact media only were used, fresh, or stored in refrigerators at 4° C., or frozen at -25° C.

Results

Large amounts of assay material were examined in preliminary studies. Findings on 25 batches of flask culture fluids and the same number of pools of roller-tube cultures are recorded. Statistical evaluation of the results was made on the basis of Fisher's recommendations (12). Duplicate, triplicate,

ill

in SE

de

SI 0 tı

is to e tl

TABLE I MEASUREMENTS OF TURBIDITY*

Medium or other material	Turbidity, units/ml.	Contact at 37° C.	Turbidity, units/ml. (error ±2.5%)
1. Flask culture of monkey kidney fragments			
134 (T alone)	10	5 days	235
148 (T + 100 mgm. cysteine/liter)	20	5 days	185
697 (synthetic mixture (20))	10	5 days	50
169 (E + 100 mgm. glycine/liter)	20	26 days	760
2. Roller-tube tissue culture of trypsinized kidney	y cortex		
199 (synthetic mixture (37)) with Hanks' base	15	_	
199 + 0.5% hs + 0.19% NaHCO ₃ (pool of 100 tubes)	90	6 hr.	90
66 66	90	6 days	78-100 2 batches
46 46 66	90	14 days	200 2 batches
199 wash fluid (7 day culture, pool of 100 tubes), no hs, + 0.19% NaHCO ₃	15	1 day	43-90 2 batches
" " "	15	3 days	60
597 + 0.19% NaHCO ₃ , no hs (14 day culture, two fluid changes—6 days with 3rd fluid, pool of 100 tubes)	25	6 days	95
597 + 2% hs + 0.19% NaHCO ₃ (no fluid change for 14 days)	387	14 days	467
3. Standard mixtures and kidney extracts			
0.1% purified serum albumin (bovine) in 199 0.05% serum albumin + 0.05% PNA + 0.05% DNA in 199		=	265 150
0.1% serum albumin in 697	_		225
1% homogenate of fresh frozen kidney cortex in water	-	6 min. homo- genate in War- ing Blendor	375
10% extract of fresh kidney cortex in water		3 min. blending, centrifuged 20 min., mean of 4 assays on pool of 10 kid- neys	1427
10% extract of fresh kidney cortex in 597		66 66	1532
5% extract of acetone-dried kidney in water		10 min. blend- ing, centri- fuged 10 min.	25
5% extract of acetone-dried kidney in 635		ii ii	115

Abbreviations: T, Tyrode solution; E, Earle solution; hs, horse serum.

*0.2 ml. medium or contact medium diluted 10-fold with 1% NaCl, 2.5 ml. 5% sulphosalicylic acid, and 0.5 ml. 20% acacia solution. Turbidity read after 10 min. at room temperature, on Klett-Summerson photocolorimeter, with filter No. 42. Blank values with or without phenol red were deducted (35).

and on some pools quadruplicate assays and determinations were run and mean values taken for the calculations. The average experimental error was illustrated in the tables.

es.

ols

as

te,

(e)

Table I presents typical turbidimetric values of contact media. An increase in turbidity index as compared to that for unused synthetic media was observed. More complex nutrients showed lower values, suggesting that cell death and autolysis may be responsible at least in part for the increase of suspended material. The known sparing effect (10) of amino acids, especially of cysteine and glycine, was confirmed. Linearity was observed with time and turbidity in some incomplete media (group 1). The addition of horse serum to the complex synthetic mixture 199 increased the initial turbidity. There is a slight fluctuation during six days' incubation due partly to dilution effect, to catabolism of the horse serum proteins, and partly to some increase in turbidity. Tubes without fluid change after 14 days, on the other hand, exhibit doubling of the original figures. There was 14% difference between the two 14-day-old tubes with 597 (one with, the other without replacement of the nutrient); but fluids of similar age in 199 showed about 55% higher turbidity. Washing fluids also contain a high amount of eluted material which is due in part to horse serum and in part to cell constituents. Similar behavior was observed in tissue cultures made up with medium 597. Experiments with standard mixtures and known kidney homogenates or extracts (group 3) illustrate the values given by some known amount of substances suspended in synthetic media or water. The solubility is parallel with the composition of the medium. It is interesting to see the difference between water and a synthetic medium and between a less and more complex nutrient mixture. Further, fresh, in contrast with acetone-dried, tissue is more highly soluble in the less complex medium 635 (20) than in 199. One per cent kidney cortex homogenate exhibited about five times higher turbidity than the supernatant of 14-day-old tubes; this gives a general idea of the amounts of materials present.

Figs. 1 and 2 show a few examples, using a sensitive and quick tool, and reveal an increasing general absorption of ultraviolet light in contact media, and increased or new maxima, between 2500 and 2900 Å, due to proteins, NA, or nucleoproteins (27). The indicator (phenol red) can be stabilized by the addition of $0.3 \, \text{ml}$. $0.2 \, N \, \text{HCl}$ (51). The differential ultraviolet spectra (Fig. 2) illustrate the difference between fresh and used nutrient fluid caused by enrichment of the latter by cell components. Unused medium 199 served as reference solution.

The activity of the various enzymes is illustrated in the following tables and figures. Table II presents typical findings of *phosphatase* assays in used media. There is a definite difference between the two main groups, but CM of varying composition present quantitative differences. More complex nutrients exhibit less intense phosphatase activity, the alkaline monoesterase being generally more active. The activating effect of various amino acids, or of varying concentrations of the same, may be seen in this and in other tables

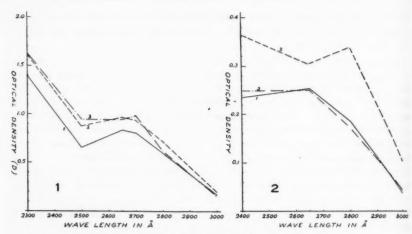


Fig. 1. Ultraviolet light absorption of M and CM (roller-tube cultures).

= 2 ml. of M 199, 2 ml. of distilled water. = 2 ml. of CM 199, 5 days' contact plus 2 ml. of distilled water. - = 2 ml. of CM 199, "wash", 1 day contact, plus 2 ml. of distilled water.

Reference: distilled water.

Fig. 2. Differential ultraviolet spectra of CM (roller-tube cultures).

--- = CM 199, "wash", 14 days' contact, 1 ml., plus 3 ml. of distilled water.
- = 1 ml. of CM 199, 6 days' contact, plus 3 ml. of distilled water.
- = 1 ml. of CM 199, "wash", 3 days' contact, plus 3 ml. of distilled water.

Reference: 1 ml. M 199 plus 3 ml. distilled water.

(group 1). The roller-tube type of cultures exhibit lower activities and tend to show only trace activity in the wash fluids (group 2). Both enzymes, but especially the acid phosphatase, diminished in activity when the six-hour and six-day figures were compared. On the eighth day a rise of alkaline phosphatase was noted, but the acid phosphatase was still of very low activity. Wash fluids even after seven days' contact (which is unusual during the procedure) exhibit low phosphatase values. On the other hand the fresh nutrient, which replaced the washing fluid, showed about six times higher alkaline phosphatase activity than acid phosphatase after six hours' contact Whether this represents the true ratio of the enzymes present or is due to the inhibition of the acid phosphatase by medium 199, and in lesser degree by 597, and simultaneous activation of the alkaline phosphatase by the same medium (consequently found in other assays (31, 32)) cannot be decided at this time. With medium 597 the enzyme activity increased in the supernatant of 14-day-old cultures, as compared with the six-hour values of the As in medium 199, the difference between the acid and alkaline phosphatase activity was still threefold in favor of the latter, which is less than the difference at eight days. The ratio of alkaline to acid phosphatase is higher than the ratio observed in normal tissue culture homogenates (31) and in surviving normal kidney cortex (32). The effect of the horse serum added is shown in the column for media (Table II, group 2).

1 1 6 -2 -1

TABLE II

ACID AND ALKALINE PHOSPHATASE IN MEDIA AND CONTACT MEDIA*

			Increas	e in IP, μgm.	/ml., mean ±	% error	
Supernatant of tissue culture in medium			Acid phosphatase		Alkaline phosphatase		
		M	CM	M	CM	Contact with tissue at 37° C.	
1. Flask culture	s of monk	ey kidney fr	agments				
134 (T alone)			0.01 ± 0.5	0.6 ±1	0.02±0	4.23±0	5 days (mean of dupli
148 (T + 1 gm	. cysteine	/liter)	0.02 ± 1.0	$\textbf{1.83} \pm \textbf{0}$	$\boldsymbol{0.02 \pm 0.25}$	10.0±0	5 days (mean of dupli cates)
697 (synthetic i	mixture (20))	0.26±0	1.40±0	0.21 ± 3	6.0±0.5	5 days (2 batches, mean of 4 assays)
2. Roller-tube ti	ssue cultu	re of trypsin	ised monkey	kidney cortex			
199 (synthetic 0.5% hs + 0 tubes pooled)	1.19% Na	(37)) + HCO ₂ (100	0.24±0.25	1.42 ± 2	0.21 ± 0.30	3.2 ±1	3 to 6 hr. (mean of 2 batches with 4 assays)
"	44	44	$\textbf{0.23} \pm \textbf{2}$	0.42 ± 1	$\boldsymbol{0.23 \pm 0.5}$	$1.0\ \pm 0$	6 days (mean of : batches, 4 assays)
**	46	46	0.24 ± 0	0.46 ± 0	0.21 ± 1	$\textbf{2.88} \pm \textbf{0}$	8 days (mean of assays)
199 WF, no ha	+ 0.19	% NaHCO	0	0.10 ± 0	0	0.1 ±0.5	
Supernatant o	f 14-day		0	$\textbf{0.03} \pm \textbf{1}$	0	0.39 ± 2.5	7 days with wash fluid (mean of 2 assays)
199, no hs + 0 fluid changes ture (100 tub	.19% Na . 3rd fluid	HCO₃ after I, 8 day cul-		0.11±1	0	0.60±2	6 hr. with 3rd fluid (mean of 2 assays)
597, no hs - supernatant culture, 2 flui	+ 0.19% of 14-day	NaHCOs, y-old tissue		0.47 ± 1.5	0	1.54±5	6 days with 3rd fluid (mean of 2 assays)

Abbreviations: As in Table I. Also: M, medium; CM, contact medium; IP, inorganic phosphate; WF, wash fluid. *2 ml. M or CM, 6 ml. acid substrate (pH 5) or 8 ml. alkaline substrate (pH 10.9), Mg^{++} 0.003 moles in alkaline system; after four hours at 37° C. add 2 ml. TCA (30%). Half filtrate taken for IP determination (43, 13, 30).

TABLE III

er.

ater.

ater.

end

but

and hosrity. the resh gher act. the by ame d at perthe line

less tase (31)rum

5-NUCLEOTIDASE AND SIMPLE NUCLEOTIDASE IN CONTACT MEDIA*

	Increase in II	P. µgm./ml. of	system, mean	± % error	
	5-Nucleo	5-Nucleotidase		leotidase	-
Medium	M	CM	M	CM	Contact at 37° C.
1. Flask culture of monkey kidney f	ragments				
169 (E + glycine, 100 mgm./liter) 0	3.46 ± 0	0	0.38 ± 2	5 days (mean of dupli- cates)
159 (E + glycine, 1 gm./liter)	0	2.50 ± 0.5	0	2.10±0	44 44
697 (synthetic mixture)	0.26 ± 2	1.70±0	0	0.87±1	5 days (mean of 4 assays)
2. Roller-tube culture of trypsinized	kidney cortex				
199 (synthetic mixture) + 0.5% h + 0.19% NaHCO ₃ (100 tube pooled)		0.27 ± 1.5	0.04±0	0.10±1	6 hr., 2 batches (mean of 8 assays)
44 46	0	0.35 ± 0	0	0.10 ± 2	6 days, 2 batches (mean of 8 assays)
68 66 66	0	1.39 ± 1	0	0.2 ±1	8 days (mean ef dupli- cates)
199 wash fluid, no hs + 0.19 NaHCO ₃	% 0	0	0	0.10±0	16 hr. (mean of dupli- cates)

*5-Nucleotidase assays: 0.5 to 2 ml. M or CM, 1.5 to 6 ml. substrate, 0.1% muscle adenylic or inosinic acid in 1.7 M veronal-acetate buffer, pH 8.5, Mg++, 0.02 moles final concentration. Incubation two hours at 37° C. Precipitation with 2 ml. 30% trichloroacetic acid. Half filtrate laken for IP determination. Enzyme (Enz.) and substrate (Sub.) controls run parallel (40, 17, 21, 30). Simple nucleotidase assays: Enz.:Sub. ratio 1:10. Sub.: equimolar mixture of 4 ribonucleotides, 0.1% concentration in 1.7 M veronal-acetate buffer, pH 8.5, Mg++, 0.02 M final conc. Procedure as above (30, 47).

Pentanucleotidase (Table III) is much more active in the contact media of flask cultures, tending to be less active in complex media, and such activity is in general of a higher order than that of simple nucleotidase (group 1). The nucleotidase activity as a whole was generally slight in the roller-tube group, though the 5-nucleotidase levels were relatively high. In eight-day-old fluids the original value was quadrupled for the former and doubled for the latter. Further investigation of this enzyme revealed that there was always less activity encountered in the supernatant of normal tissue culture homogenates (31) than in the tissues or in whole homogenates. This gives special significance to the study of this enzyme, because its abundance in the medium may be a sign of cell destruction. (Compare medium 169 to 697 in group 1 and results in group 2.) It is a resistant enzyme (21) activated by most of the synthetic nutrient mixtures (31, 32, 34) which makes its detection easier. The only medium which caused some spontaneous hydrolysis of 5-nucleotides was the 697. Horse serum in the amount used did not confer any nucleotidase activity to the tissue culture fluid in roller tubes.

Two types of *ribonuclease* (Table IV) and two types of desoxyribonuclease (Table V) with various pH optima were detected in the used media. The ribonuclease was generally very active (Table IV, group 1) and was also detected in about the same amount in the trypsinized tubes, if the amount introduced by horse serum (group 2) is not considered. The "acid" enzyme was of significantly higher activity in both groups, an incomplete medium (E with 1 gm./liter glycine) (group 1) exhibiting the highest activity in the whole

TABLE IV
RIBONUCLEASE IN CONTACT MEDIA*

		Increase in TASP, μ gm./ml. of system, mean of duplicates $\pm \frac{G}{G}$ error		
Medium	Contact	At pH 5	At pH 7.6	
1. Flask culture of monkey kidney fragments				
159 (E + 1 gm. glycine/liter)	0	No change	1.0 ± 2	
	5 days	18.4 ± 2	10.9 ± 1	
169 (E + 100 mgm. glycine/liter)	0	0.26 ± 5	1.0 ± 2	
16 16 16	5 days	13.3 ± 1	8.0 ± 0	
697 (synthetic mixture (20))	0	0.52 ± 0	1.3 ± 1.5	
66 66 66	5 days	13.8 ± 0.5	7.2 ± 2	
2. Roller-tube cultures of trypsinized monkey	kidney cell	ls		
199 (synthetic mixture)	0	0	0	
199 + 0.5°; hs + 0.19°; NaHCO ₃	0	5.8 ± 0.5	2.8 ± 0.8	
46 46 46	6 hr.	10.6 ± 5	5.2 ± 0.5	
46 44 46	1 day	9.20 ± 1	3.5 ± 2.0	
4.6 0-6 0-6	6 days	10.7 ± 0	8.6 ± 5	
199 wash fluid, no hs + 0.19° NaHCO3	1 day	5.0 ± 0.25	1.5 ± 1	

^{*}Enzyme (Enz.): 0.5 to 2 ml. medium or contact medium. Substrate (Sub.): 0.1% polymer pentose nucleic acid in 0.1 M acetate buffer, pH 5.0, or 0.12 M borate buffer, pH 7.6. Composition. Enz.:Sub. 1:2. Incubation four hours at 37° C. Precipitate with 2 vol. 5% trichloroacetic acid containing 0.25% uranylacetate. Half filtrate digested for total acid soluble phosphorus (TASP) determination (25, 28).

E

e

S

2 i-

e S

ic

v

ie J.

98

1e d

ed of

th

ole

m,

5

8 5 0

mei ion acid SP) series. The difference between the two enzymes varied with the various media, depending on differential activation or inhibition, as suggested by the work with crystalline ribonuclease (34). The largest difference was found in Earle's solution plus glycine (1 gm./liter) in group 1 and in one-day-old contact medium of group 2. On the other hand the closest similarity was found in Earle's solution plus 100 mgm. glycine/liter and in six-day-old roller tubes with 199. The alkaline ribonuclease exhibited a rise from one-to six-day-old contact media (group 2). The activity of "wash fluid" was slightly lower than that of the medium plus horse serum. Many media of group 1 caused some spontaneous increase of TASP (maximum 18% of system) which may be due to salt and pH effect (25). On the other hand, 0.5% horse serum plus bicarbonate adds about 50% to the ribonuclease activity in roller tubes (group 2) until the first fluid change. This is washed away by the washing medium after six days.

Desoxyribonuclease, as illustrated in Table V, also showed higher values in the acid range and significantly higher activity in the first main group. desoxyribonuclease values in group 1 vary with the media used. With higher amino acid content higher values were obtained; derivatives containing SHgroups were especially very effective.

TABLE V DESOXYRIBONUCLEASE ACTIVITY IN CM (VISCOSIMETRIC TECHNIQUES)*

	Amount	Activity measured by % decrease relative viscosity of system, error ± 3		
Medium	used, ml.	At pH 5	At pH 7	
1. Flask culture (pools)				
135 + (T + 100 mgm. glycine/liter)	0.3	0.5	.1.0	
CM 135 (5 days)	0.3	15.0	2.0	
159 + (E + 1 gm. glycine/liter)	0.3	No change	No change	
CM 159 (5 days)	0.3	16.0	10.0	
137 + (T + 1 gm. glycine/liter)	1.0	. 1.0	0.6	
CM 137 (5 days)	1.0	21.0	21.0	
CM 148 (5 days) + T + 1 gm. cysteine/ liter	1.0	37.0	23.0	
2. Roller-tube culture (pools)				
199 + 0.19% NaHCO ₂ + 0.5% hs	1	No change	No change	
CM 199 + 0.19% NaHCO ₂ + 0.5% hs (6 hours)	0.3	0.7	No change	
CM 199 + 0.19% NaHCO ₃ + 0.5% hs (6 days)	1.0	4.0	5.0	
CM 199 WF (1 day), no hs + 0.19% NaHCO ₃	1.0	No change	No change	

Abbreviations: As in previous tables. "Enzyme: 0.3 to 1 ml. M or CM. Substrate: 0.1% highly polymerized desoxyribonucleic acid in 0.14 M veronal-acetate buffer of pH 7 and pH 5, Mg++ 0.003 moles and 0.01% gelatin. Flow time of this mixture is taken three times in an Ostwald pipette, then after thorough mixing with ingredients time of flow is measured and the readings repeated after one and two hours' incubation at 30° C. Per cent drop of relative viscosity expresses the depolymerase activity (36, 44, 29).

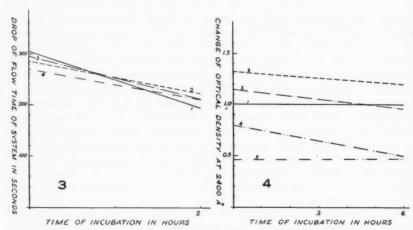


Fig. 3. Desoxyribonuclease in CM measured by decrease of the flow time of the system. Curve 1 1 ml. of CM 148 plus 4 ml. of 0.1% DNA in veronal-acetate buffer, pH 5. Curve 2 -1 ml. of CM 148 plus 4 ml. of 0.1% DNA in veronal-acetate buffer, 1 ml. of CM 137 plus 4 ml. of 0.1% DNA in veronal-acetate buffer, Curve 3 1 ml. of CM 137 plus 4 ml. of 0.1% DNA in veronal-acetate buffer, Curve 4 pH 5.

Fig. 4. Cholinesterase in CM measured by decrease of ultraviolet light absorption of the system at 2400 Å.

2 ml. of M 199 plus 2 ml. of benzoylcholine, $10^{-7}\ M$ in phosphate buffer pH 7.4. Curve 1 2 ml. of CM 199, "wash", 1 day contact plus 2 ml. of benzoyl-

Curve 2 - choline, 10-7 M in phosphate buffer pH 7.4

2 ml. of CM 199, 3 days' contact, plus 2 ml. of benzoylcholine, 10⁻⁷ M in phosphate buffer pH 7.4. = 0.5 ml. of CM 135, 6 days' contact, plus 3.5 ml. of benzoylcholine,

10⁻⁷ M in phosphate buffer pH 7.4.

2 ml. of benzoylcholine substrate plus 2 ml. of phosphate buffer, pH 7.4.

Alkaline and acid desoxyribonuclease were of equal activity in Tyrode solution plus glycine: a much lower alkaline desoxyribonuclease activity was found with Earle's solution plus the same amount of glycine. Evidently the difference in salt mixtures is responsible for this finding (32). Otherwise the acid desoxyribonuclease was much more resistant than the other nuclease, which is generally known to be a very sensitive enzyme (36, 44). The moderate activities observed in six-day-old fluid of roller-tube culture (with the unusual finding of higher alkaline desoxyribonuclease activity) may mean that there is less cell destruction than in the flask culture; but the depressive activity of medium 199 on these depolymerases was also observed in this and in later work (31, 32). Medium 597 was less inhibitory, so some of the pyrimidines or purines may be responsible for the suppression of the desoxyribonuclease (34). Fig. 3 illustrates the drop in relative viscosity by the decrease of flow time in seconds. Both the percentage drop of relative viscosity or the actual drop of flow time are suitable expressions of nuclease activity, as shown previously by total acid soluble phosphorus determinations (29). The highest value in group 1 was found with CM 148 (E plus cysteine), and in group 2 with six-day-old fluids of roller tubes, illustrated in Table V and Fig. 3. Because of the specific activity of this amino acid (16), Tyrode solution plus glycine (1 gm./liter) was the only fresh medium that caused some depolymerization.

Pseudocholine esterase, xanthine oxidase, and nucleoside phosphorylase were also detected in contact media, but to conserve space only one of them is illustrated. Fig. 4 shows the decrease of optical density of benzoyl choline substrate when incubated with a small amount of used medium, under the conditions listed under the figure. This change is due to the activity of pseudocholine esterase (24).

The linearity with turbidity in general was not strict, because only one part of the substances giving opalescence by these techniques is protein material. The correlation with protein nitrogen and enzyme activity on the other hand showed a more strict relationship in the few cases investigated. Finally, to prove the intrinsic origin of these enzymes, the only source, apart from the cells, namely the horse serum, was also assayed in saline, in the concentrations routinely used (0.5 to 10%). We have not included this series of work in separate tables, but have already illustrated some similar findings in connection with the enzyme assays in the roller-tube series. The phosphatases and the ribonuclease found in the horse serum may represent measurable additions to the enzyme activity of the contact media, when not inactivated. In connection with the kinetics of the enzymes studied, we have quoted some examples of activation by amino acids (compare 134 and 148 in Table IV), and of

TABLE VI

EFFECT OF COLCHICINE ON PHOSPHATASES IN CM 697*

(Incubation, 16 hr. at 37° C.)

Medium	Enzyme	Increase IP, µgm. of system/ ml. M or CM
697 (synthetic mixture (20))	Acid phosphatase	No change
697 (synthetic mixture (20))	Alkaline phosphatase	No change
697 + colchicine 0.0058 μgm./ml. (5 days' contact)	Acid phosphatase	No change
597 + colchicine 0.0058 μgm./ml. (5 days' contact)	Alkaline phosphatase	No change
CM 697 (5 days' contact)	Acid phosphatase	13.1
CM 697 (5 days' contact)	Alkaline phosphatase	71.1
CM 697 (5 days' contact) + colchicine 0.0058	Acid phosphatase	9.6
CM 697 (5 days' contact) + colchicine 0.0058 µgm./ml.	Alkaline phosphatase	59.9

Abbreviations: As in previous tables.

^{*1} ml. M or CM, 9 ml. Sub., colchicine 0.0058 µgm./ml. final concentration. Phosphatase determinations by Shinowara's method (43).

inhibition by various components of the medium. The effect of other substances, colchicine for instance, included in some of the nutrient fluids in flask culture assays (medium 140, etc.)⁴ was also investigated. Table VI presents a few experiments with this drug added in very low concentration to the systems prepared with contact media. Under these circumstances both acid and alkaline phosphatase were definitely inhibited: 30 and 20% respectively.

Discussion

Analysis of contact media by the methods applied may furnish relevant information, without interfering with cell life, by simple withdrawal of samples of the nutrient fluid. High turbidity values and enzyme activities in the first days after explantation may be the consequence of cell death and autolysis due to the traumatism (2) suffered by the tissues during the procedures. Incomplete media do not support survival of cells, except for short periods of time (10, 11), although they may be suitable for virus growth (3, 19). ultimate goal of the present experiments, virus cultivation, was successful although the conditions of growth were not always adequately controlled. The examination of this routine material revealed many interesting findings and may be repeated with rigorous techniques to clarify a few points. presence of enzymes in the supernatant is significant, because it represents a constant loss of specialized tissue constituents at each fluid change. The question whether it is a pathological, physiological, or a physicochemical phenomenon cannot be decided for the moment. We are inclined to speculate that all three factors together play a part. In complete media such as 697 (20) and 199 (37) the cell death alone was not sufficient to account for the The duplication of tissue mass was observed in many of the flask cultures (31) from 5 to 11 days, and in the majority of roller-tube cultures at 14 days, by homogenate techniques checked by turbidimetry. This means that growth occurred in these cases. The investigation of a possible secretion or extrusion of enzymes during mitosis, or under other conditions, would be an interesting task. On the other hand the presence of structurally firmly bound enzymes (47) in the supernatant in larger amount has to be regarded as a sign of cell disintegration in mass.

There is great variation in the ability of various media to extract cell constituents as compared to water. We feel that this question requires special attention, because the findings on homogenates may not be directly compared with intact cells. Further, there may be variable sensitivity during mitosis or in young and old cells, which factors have to be carefully considered. The differences observed between the two types of tissue cultures are tentatively explained by the following factors: difference in size (0.062% for trypsinized cortex and 1% whole kidney wet weight for the flask cultures); difference in solubility in various media; whole kidney homogenates give lower turbidity

⁴Unpublished assays, personal communication of Dr. A. E. Franklin.

values than kidney cortex (32); differences in physiological value of the media; and finally the trypsinization, which represents a substantial source of enzyme losses (31).

Many authors have examined the question of complete and incomplete media (2, 10, 11, 18, 3, 19). Older (42) and newer data (18) emphasize the wide range of tolerance of the tissues *in vitro*, with regard to survival and multiplication. There was perhaps less cell destruction in our experience, when complex media were used, but even these media have to be carefully investigated for the interesting properties revealed during these studies, namely for differential activation and inhibition of various enzyme systems (31, 32, 34). All these findings open up the possibility of interesting approaches for enzyme studies in cultivated cells (31). The ferment systems described in used media are not necessarily in the same ratio as in normal cells (32). Because of their differences in kinetics the problem is more complicated. In an extensive comparative study in roller-tube tissue cultures, where the conditions of growth were more adequate, the distribution of enzymes between supernatant and whole tissue culture homogenates and sediment were assayed and the results will be reported (31).

The findings described suggest that some enzymatic cell functions are on a lower level in the explants and that this lower function may be an important factor in infectivity with poliomyelitis virus. The depletion of biocatalysts of tissues, although temporary, may be of paramount significance. It may be easier to direct these cells into some specific synthetic and metabolic pathway, that is, into virus production. The correlation of depletion of cells (due to incomplete nutrients) and virus infection was investigated by Burr *et al.* (3) and by Hare and Morgan (19). Our working hypothesis, however, is basically different from theirs and we will further develop this line with clinical and experimental evidence (31, 32).

Conclusions

Among others, some enzyme systems concerned with nucleic acid metabolism were demonstrated in the tissue culture fluids. Depletion of these highly specialized constituents from the tissues means that the functional integrity of the explant is different from the original; this state may render the cells susceptible to poliomyelitis virus infection and facilitates virus production.

Acknowledgment

The material and moral support of Dr. R. D. Defries, Director of the School of Hygiene and the Connaught Medical Research Laboratories, and of Dr. M. H. Brown, Professor and Head of the Department of Hygiene and Preventive Medicine, are gratefully acknowledged. Special thanks are due for the greatest co-operation of Dr. A. E. Franklin, Dr. D. R. E. MacLeod, Dr. G. H. Macmorine, Mr. F. T. Shimada, and Mrs. Stephany Schenk of the Connaught Medical Research Laboratories for the generous supply of the assay material used in this study. We also thank Dr. W. Kalow, of the Department of

Pharmacology, University of Toronto, for his help and advice during cholinesterase assays. The work was carried out mostly with the technical help of Mr. Louis Szendy and Mrs. Katherine Vlossak, and in part with that of Mr. Joseph Kovacs. Thanks are also extended to Miss R. M. Briggs, Librarian of the Connaught Medical Research Laboratories, for her help in the preparation of this manuscript.

References

- 1. ALBANESE, A. A., IRBY, V., and SAUR, B. J. Biol. Chem. 166: 231. 1946.
- BRUES, A. M., RATHBURN, E. N., and COHN, W. E. J. Cellular Comp. Physiol. 24: 155. 1944.
- Burr, M. M., Campbell, M. E., Morgan, J. F., and Nagler, F. P. Can. J. Microbiol. 1:158. 1941.
- 4. CARREL, A. J. Exptl. Med. 15:516. 1912.
- 5. Dulbecco, R. and Vogt, M. J. Exptl. Med. 99:167. 1954.
- DUNCAN, D., FRANKLIN, A. E., WOOD, W., and RHODES, A. J. Can. J. Biochem. Physiol. 31:75. 1953.
- 7. EAGLE, H. J. Biol. Chem. 214:839. 1955.
- 8. ENDERS, J. F., WELLER, T. H., and ROBBINS, F. C. Science, 109:85. 1949.
- 9. FARRELL, L. N., WOOD, W., FRANKLIN, A. E., SHIMADA, F. T., MACMORINE, H. G., and RHODES, A. J. Can. J. Public Health, 44:273. 1953.
- 10. FISCHER, A. Acta Physiol. Scand. 2:143. 1941.
- FISCHER, A., ASTRUP, T., EHRENSVARD, G., and OEHLENSCHLAGER, V. Proc. Soc. Exptl. Biol. Med. 67: 40. 1948.
- FISHER, R. A. Statistical methods for research workers. 8th ed. Oliver & Boyd, Ltd., London. 1941.
- 13. FISKE, C. H., and SUBBAROW, Y. J. J. Biol. Chem. 66: 375. 1925.
- Franklin, A. E., Duncan, D., Wood, W., and Rhodes, A. J. Proc. Soc. Exptl. Biol. Med. 79: 715. 1952.
- FRANKLIN, A. E., DUNCAN, D., WOOD, W., and RHODES, A. J. Can. J. Biochem. Physiol. 31: 67. 1953.
- GREENSTEIN, J. P. and JENRETTE, W. V. Cold Spring Harbor Symposia Quant. Biol. IX: 236. 1941.
- 17. GULLAND, J. M., and JACKSON, E. M. Biochem. J. 32:595. 1938.
- 18. HANKS, J. H. J. Cellular Comp. Physiol. 31: 235. 1948.
- 19. HARE, J. D. and MORGAN, H. R. J. Exptl. Med. 99; 461. 1954.
- HEALY, G. M., FISHER, D. C., and PARKER, R. C. Can. J. Biochem. Physiol. 32: 327. 1954.
- 21. HEPPEL, L. A. and HILMORE, R. J. J. Biol. Chem. 188: 665. 1951.
- 22. HULL, W. and KIRK, P. I. J. Gen. Physiol. 33: 327. 1950.
- 23. KALCKAR, H. M. J. Biol. Chem. 167: 461. 1947.
- 24. KALOW, W. J. Pharmacol. Exptl. Therap. 34:122. 1952.
- 25. King, E. J. Biochem. J. 26: 292. 1932.
- 26. KOCH, F. C. and McMEEKIN, T. L. J. Am. Chem. Soc. 46: 2066. 1924.
- 27. Kovacs, E. Can. J. Med. Sci. 31: 437. 1953.
- 28. Kovacs, E. Can. J. Biochem. Physiol. 32:526. 1954.
- 29. Kovacs, E. J. Pediat. 45: 569. 1954.
- 30. Kovacs, E. J. Pediat. 47: 340. 1955.
- 31. Kovacs, E. Unpublished.
- 32. Kovacs, E. Unpublished.
- 33. Kovacs, E. Unpublished.
- 31. Kovacs, E. Unpublished.
- 35. LOONEY, J. M. and WALSH, A. I. J. Biol. Chem. 130: 635. 1939
- 36. MACCARTY, M. J. Gen. Physiol. 29: 123. 1946.

- 37. MORGAN, J. F., MORTON, H. J., and PARKER, R. C. Proc. Soc. Exptl. Biol. Med. 73: 1. 1950.
- PARKER, R. C., HEALY, G. M., and FISHER, D. C. Can. J. Biochem. Physiol. 32: 306. 1954.
- PARKER, R. C. Methods of tissue culture. 2nd ed. P. and B. Hoeber, Inc., New York. 1950.
- 40. Reis, J. Enzymologia, 5:251. 1938-39.
- 41. ROBBINS, F. C., WELLER, T. H., and ENDERS, J. F. J. Immunol. 69: 673. 1952.
- 42. Rous, P. J. Exptl. Med. 18:183. 1913.
- 43. Shinowara, G. Y., Jones, L. M., and Reinhardt, H. L. J. Biol. Chem. 142:921. 1942.
- 44. Siebert, G., Lang, K., Lucius-Lang, S., Herkert, L., Stark, G., Rossmüller, G., and Jöckel, H. Hoppe-Seyler's Z. physiol. Chem. 295: 229. 1953.
- 45. SIMMS, H. S. and STILLMAN, N. P. J. Gen. Physiol. 20: 603. 1937.
- 46. SOBEL, A. E., MAYER, A. M., and GOTTFRIED, S. P. J. Biol. Chem. 156: 355. 1944.
- 47. STERN, H., ALLFREY, W., MIRSKY, A. E., and SAETRENS, H. J. Gen. Physiol. 25: 539, 1952.
- 48. WHITE, P. R. Growth, 10:231. 1946.
- 49. WILSON, H., JACKSON, E. B., and BRUES, A. M. J. Gen. Physiol. 25:689. 1941-42.
- WOOD, W., FRANKLIN, A. E., DUNCAN, D., and RHODES, A. J. Proc. Soc. Exptl. Biol. Med. 81: 414. 1952.
- 51. YOUNGNER, J. S. Proc. Soc. Exptl. Biol. Med. 85: 202. 1954.

Symposium on the Chemistry and Physiology of Phospholipids

This symposium was sponsored by the Biochemistry Subject Division of the Chemical Institute of Canada and was held at the University of Western Ontario, London, Ontario, on October 12th and 13th, 1955. Dr. A. M. Wynne of the University of Toronto acted as Chairman of the session on the chemistry of phospholipids and Dr. W. R. Bloor of the University of Rochester was Chairman of the session on the metabolism and function of phospholipids. The symposium was organized by Dr. R. J. Rossiler of the University of Western Ontario.

THE SYNTHESIS OF GLYCEROLPHOSPHATIDES1

By E. BAER

I would like to express my thanks to the Committee who organized this symposium for their kind invitation to open the proceedings by discussing the synthetic work that has confirmed and extended our knowledge of the structure of phosphatides. It is not my intention to attempt a complete survey of the efforts along these lines. For obvious reasons I propose to confine my remarks to our more recent work concerning the synthesis of glycerolphosphatides.

During the greater part of the past twenty years it has been one of the aims of our laboratory to develop methods that permit the synthesis of both enantiomeric forms of asymmetrically substituted glycerol derivatives in an optically pure state. Our main objective, from the beginning, has been to achieve the synthesis of naturally occurring phosphatides with a view to determining their structure and configuration. Since there existed good experimental evidence that the natural glycerolphosphatides occur in optically active form, the synthetic phosphatides, to be fully identical with the natural ones, had to be obtained in the enantiomeric form of the natural product. This required the development of procedures that yield the pure enantiomeric forms of the glycerolphosphatides. These procedures, if possible, should not rely on the resolution of racemates, since this process does not always give pure enantiomers. Furthermore to be able to assign a configuration to the synthetic glycerolphosphatide, the synthetic procedure had to reveal the stereochemical relationship of the phosphatide and its intermediates to the D- and L-glyceraldehydes, the stereochemical compounds of reference. This became possible when Hermann O. L. Fischer and I succeeded in 1937 in devising a method for the synthesis of both the D- and L-acetone glycerols. Since both have become key substances in the synthesis of optically active glycerolphosphatides and provide the stereochemical link from the phosphatides to D- and L-glyceraldehyde, I would like to show you briefly how they are obtained. This will also reveal to you some of the work involved in preparing the starting materials for our syntheses (Reaction Scheme 1).

To obtain the D-acetone glycerol, commercially available D-mannitol is acetonated in the 1,2 and 5,6 positions by means of acetone and zinc chloride.

¹Manuscript received December 1, 1955.

Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ont. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955.

Oxidative cleavage of the 1,2,5,6-diacetone D-mannitol with lead tetraacetate yields 2 moles of acetone D-glyceraldehyde that on reduction with hydrogen in the presence of nickel catalyst form 2 moles of D-acetone glycerol. The synthesis of L-acetone glycerol, which in its later stages is identical with that of D-acetone glycerol, is considerably more time consuming since the L-mannitol has to be prepared from L-arabinose via the L-mannic acid lactone (Reaction Scheme I).

The D- and L-acetone glycerols are phosphorylated either directly and then yield the fatty acid free intermediates of the phosphatides, that is, glycerylphosphorylethanolamine, glycerylphosphorylcholine, and glycerylphosphorylserine, or they serve to prepare optically active alpha, beta-diglycerides which in turn on phosphorylation yield the phosphatides and their fatty acid-containing intermediates.

Reaction Scheme II describes the synthesis of the optically active diglycerides by the method of Sowden and Fischer (7). The use of the optically pure acetone glycerols or alpha, beta-diglycerides is responsible for the optical purity of our synthetic phosphatides. Other contributing factors are the use of suitable phosphorylating agents such as phenylphosphoryl dichloride or diphenylphosphoryl chloride as both yield fewer by-products than phosphorus oxychloride; the precaution of blocking all reactive groups, with the exception of those at which the reaction is to take place, by readily removable substituents; and finally the selection of reaction conditions that do not cause the destruction of the asymmetry of the optically active glycerol derivatives.

Table I lists the phosphate esters that have been synthesized in our laboratory. As it can be seen the structure of these compounds is becoming increasingly complex with our growing experience. Starting with the synthesis of alpha-glycerophosphoric acid in 1937, we proceeded to synthesize alpha-glycerylphosphorylcholine, alpha-phosphatidic acids, alpha-bisphosphatidic acids, saturated representative members of the alpha-lecithins and alpha-cephalins, alpha-glycerylphosphorylethanolamine, phosphatidyl serine, alpha-glycerylphosphorylserine, and very recently an alpha-phosphatidyl peptide and the first fully unsaturated alpha-lecithin. Of most of these substances both optical isomers were prepared.

L-α-		L-α-		
1. Glycerophosphoric acid	(1937)	7. Glycerylphosphorylethanolamine	(1953)	
2. Glycerylphosphorylcholine	(1948)	8. Glycerylphosphorylserine (unpublished)		
3. Lecithins (saturated)	(1949)	9. Phosphatidyl serine	(1955)	
4. Cephalins (saturated)	(1951)	10. Phosphatidyl peptides (unpublished)		
5. Phosphatidic acids	(1951)	11. Lecithins (unsaturated)	(1956)	
6. Bis-phosphatidic acids	(1952)			

REACTION SCHEME I

CH₃ O-CH₂

$$CH_3 O-CH_2$$

$$CH_4 O-CH_2$$

$$CH_4 O-CH_2$$

$$CH_5 O-CH_4$$

$$HO-C-H$$

$$HO-C-H$$

$$H-C-OH$$

$$H-C-O$$

$$H_2C-O$$

$$CH_4$$

$$H_3C-O$$

$$CH_4$$

$$H_4$$

H₂C--OH

Ni, H.

H-C=0

H--2=0

- L-Mannitol ---- L-Mannonic lactone L-Arabinose ---

1,2,5,6-Diacetone L-mannitol

L-Acetone glycerol

Acetone 1.-glyceraldehyde

REACTION SCHEME II

D-Alpha, beta-diglyceride

Sowden and Fischer 1941

To illustrate our synthetic methods, I would like to show you briefly our synthesis of phosphatidyl serine, although some of you may be already familiar with it through our recent publication in the Journal of Biological Chemistry (1). The synthesis was carried out by Dr. Jonas Maurukas.

The synthesis of a substance of this type is complicated by the fact that it contains two asymmetric centers, one in the phosphatidyl moiety and one in the amino acid moiety, and thus can occur in four stereoisomeric forms. Other difficulties arise by the presence of the reactive amino and carboxyl groups in serine, which, if not blocked, would interfere with the phosphorylation of the hydroxyl group of the amino acid. Reaction Scheme III shows our procedure and illustrates how the various difficulties were overcome. D-alpha, beta-distearin (II) prepared by the method of Fischer and Sowden, was phosphorylated with phenylphosphoryl dichloride in the presence of pyridine yielding a mixture of distearoyl L-alpha-glycerylphenylphosphoryl chloride (III) and tetrastearoyl bis-(L-alpha-glyceryl) phosphoric acid phenyl ester (IV). Without separating this mixture, the distearoyl L-alpha-glyceryl-

REACTION SCHEME III

D-α.β-Diglyceride

11

ar

ry

it

in

S.

yl

a-

VS

e.

n,

of

yl

yl

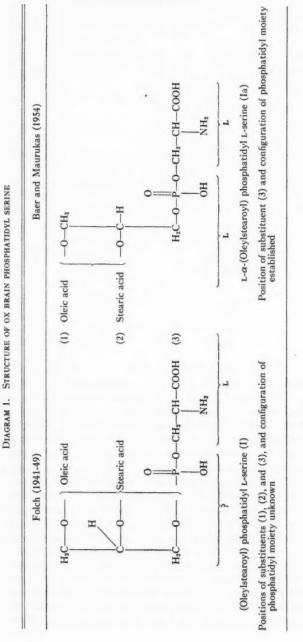
ıl-

phenylphosphoryl chloride (III) was immediately esterified with N-carbobenzoxy L-serine benzyl ester (V) yielding distearoyl L-alpha-glycerylphenylphosphoryl N-carbobenzoxy L-serine benzyl ester (VI). This compound was isolated and freed of its protective phenyl-carbobenzoxy and benzyl groups in one operation by catalytic hydrogenolysis using a mixture of platinum and palladium as catalyst. The L-alpha-(distearoyl) phosphatidyl L-serine (VII) was obtained in the form of a fine white powder in an over-all yield of 23%. By comparing the synthetic compound (VII) with the reduction product of phosphatidyl serine of ox-brain, the structure and configuration of the natural phosphate ester was established. The structure and configuration of the natural product as far as it is now known is shown on Table II.

Although during the past few years the complex lipoproteins have been intensively studied by biochemists, physiologists, and clinical investigators, little is known of their exact chemical structure. Even less seems to be known about the forces effecting a linkage of the phosphatide and protein moieties.

Recently, in the course of their investigation of the lipids of blood serum, Schrade and associates (2, 6), using a combination of one- and two-dimensional paper chromatography, reported the isolation of 13 individual lipopeptides.

TABLE II



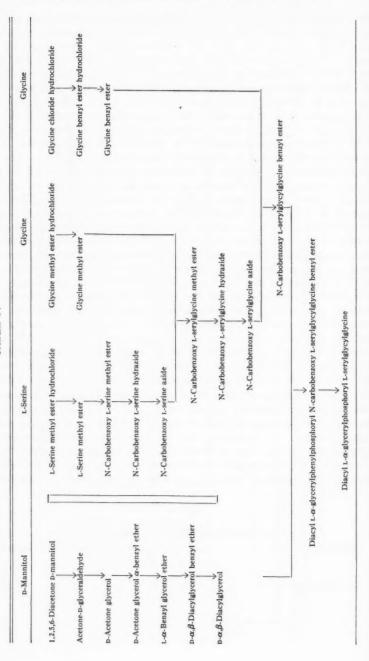
Toff et to ff it is es s s FI to F

The phosphatide-like nature of the lipopeptides suggested by their solubility characteristics seemed to be confirmed by positive reactions for choline and fatty acids, and the fact that the ninhydrin-positive spots given by lipid extracts of rats treated with P32 were radioactive. The acid hydrolysis of the individual lipopeptides and the separation of the amino acids by twodimensional paper chromatography revealed that the peptide chains contained from 3 to 11 different amino acids. In practically all of the lipopeptides a hydroxyamino acid was present, thus suggesting the possibility that the phosphatidyl moieties are linked to the peptide chains via the hydroxyl group of the hydroxyamino acid, and that the phosphatidyl peptides in fact are the phosphoric acid esters of hydroxyamino-acid-containing peptides. The most simple representative member of this class of compounds would be phosphatidyl serine whose synthesis has just been described. A few months after Schrade's publication, a paper by Bode (3), a former associate of Schrade's, appeared in which Bode claims that the evidence which seemed to support the natural existence of lipopeptides had been wrongly interpreted. However, in spite of the still controversial nature of the lipopeptides described by Schrade et al., it was felt that these compounds, existing or not, are interesting enough to warrant an extension of our synthetic work to the synthesis of a simple representative of this group. It was hoped that a knowledge of the chemical and physical properties of a synthetic phosphatidyl peptide of known structure and configuration would greatly assist in the elucidation of the structure of the more complex lipoproteins. It was thought that a phosphatidyl tripeptide with a structure as shown by Table III, that is the L-alpha-(distearoyl) phosphatidyl L-serylglycylglycine, would possess many of the chemical and physical properties of phosphatidyl peptides with longer peptide chains but would still be amenable to synthesis by our present methods.

To give you an idea of the work involved in the synthesis of even such a rather simple phosphatidyl peptide which was carried out by Dr. Jonas Maurukas, I have listed in Table IV the 22 intermediates which our present method requires. It is possible that by simplifying the synthesis of the tripeptide several steps may be eliminated. You will notice that the starting materials are the commercially available D-mannitol, L-serine, and glycine. The upper left section of the table gives the intermediates required for the

TABLE III

TABLE IV



synthesis of the alpha,beta-diglyceride, namely, 1,2,5,6-diacetone D-mannitol, acetone D-glyceraldehyde, D-acetone glycerol, D-acetone glycerol benzyl ether, L-alpha-benzyl glycerol ether, D-alpha,beta-diacylglycerol benzyl ether.

The upper right section of the table describes the synthesis of the N-carbobenzoxy L-serylglycylglycine benzyl ester. To prepare the tripeptide derivative, L-serine is converted into N-carbobenzoxy L-serine azide via L-serine methyl ester hydrochloride, L-serine methyl ester, N-carbobenzoxy L-serine methyl ester, and N-carbobenzoxy L-serine hydrazide. The N-carbobenzoxy L-serine azide was made to react with glycine methyl ester prepared from glycine. The resulting N-carbobenzoxy L-serylglycine methyl ester was converted via its hydrazide into the N-carbobenzoxy L-serylglycine azide. The reaction of this compound with glycine benzyl ester gave the N-carbobenzoxy L-serylglycylglycine benzyl ester. The final steps of the procedure are shown in greaten detail by Reaction Scheme IV.—The D-alpha,beta-diglyceride is phosphorylated with phenylphosphoryl dichloride, and the reaction product is esterified with the tripeptide derivative. The resulting phosphatidyl tripeptide derivative on removal of its three protective groups by catalytic hydrogenolysis yielded the L-alpha-(distearoyl) phosphatidyl L-serylglycylglycine.

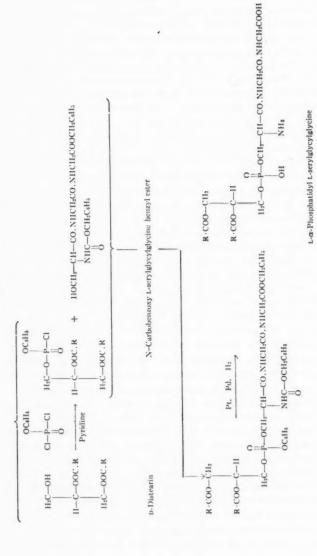
After purification of the crude material a fine white powder was obtained that gave correct analyses for the phosphatidyl tripeptide. At the present time our yield of the phosphatidyl tripeptide is still quite low and we had just sufficient material to establish its elementary composition. If more material becomes available we intend to *determine* its infrared spectrum and its X-ray diffraction pattern; to study its hydrolysis by acid, alkali, or diazomethane; and what seems to us of particular interest, to *investigate* the stability of the phosphatidic acid – peptide ester bond towards the commonly used solvents for the extraction of phospholipids from natural sources, namely boiling alcohol or boiling mixtures of alcohol and ether. This investigation, we hope, will also give some information as to the nature of the forces joining the phosphatide and protein moieties in natural lipoproteins.

I would like, now, to discuss briefly our recent and still unpublished work dealing with the synthesis of lecithins containing two identical fatty acids.

The isolation of a lecithin containing two saturated fatty acids per molecule by Lesuk and Anderson (5), and Thannhauser, Benotti, and Boncoddo (8), respectively, or two *unsaturated* fatty acids by Hanahan and Jayko (4), invalidates the hypothesis put forth by earlier workers that all natural lecithins contain one molecule each of a saturated and an unsaturated fatty acid. Some time before Hanahan and Jayko's publication appeared, work had been in progress in our laboratory to develop a procedure that would permit the synthesis of the racemic as well as both enantiomeric forms of alpha-lecithins with two identical *unsaturated* fatty acid substituents.

The procedure that we had developed for the synthesis of fully saturated lecithins by means of phenylphosphoryl dichloride cannot be used for the synthesis of unsaturated lecithins, although the phenyl esters of the unsaturated lecithins could be obtained by this method. The removal of the phenyl

REACTION SCHEME IV

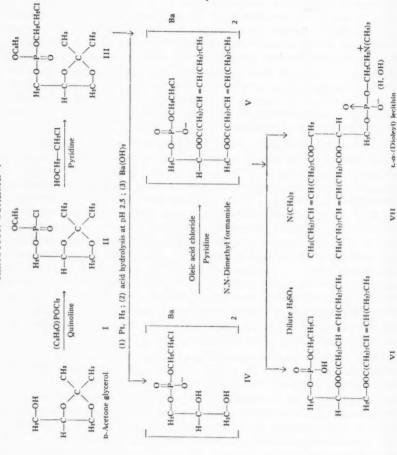


group of the unsaturated lecithin by catalytic reduction, which would be the last step in the synthesis, would cause the simultaneous reduction of the unsaturated fatty acids and thus yield as the final product a fully saturated lecithin. Since, however, in our experience, phenylphosphoryl dichloride has proved itself superior in several respects to phosphorous oxychloride as phosphorylating agent, it was deemed desirable to devise a procedure that would permit its use in the synthesis of unsaturated lecithins. Obviously in this case the removal of the phenyl group by catalytic hydrogenolysis would have to precede the introduction of the unsaturated fatty acid substituents into the glycerol moiety of the phosphatide. With this restriction in mind a sequence of reactions was devised by means of which the first synthesis of a fully unsaturated lecithin, namely, L-alpha-(dioleyl) lecithin, was achieved. Although as yet it has not been shown to be a natural product, the dioleyl lecithin was selected for synthesis because of the presence of oleic acid in many of the naturally occurring glycerophosphatides, and also in the hope of obtaining a good substitute for beef heart lecithin as an antigen component in the serodiagnosis of syphilis.

Reaction Scheme V shows our procedure for the synthesis of unsaturated alpha-lecithins which has been carried out by Dr. Buchnea. D-Acetone glycerol (I) is phosphorylated by means of phenylphosphoryl dichloride and quinoline, and the resulting acetone L-alpha-glycerylphenylphosphoryl chloride (II), is esterified with ethylene chlorohydrin in the presence of pyridine. The acetone L-alpha-glycerylphenylphosphoryl ethylene chlorohydrin (III) is freed of its protective phenyl group by catalytic hydrogenolysis and of its acetone group by a mild acid hydrolysis. The resulting L-alpha-glycerylphosphoryl ethylene chlorohydrin was isolated in the form of its barium salt (IV). A treatment of the barium salt with oleyl chloride and pyridine in anhydrous dimethylformamide gave the barium salt of L-alpha-dioleyl glycerylphosphoryl ethylene chlorohydrin (V), which on treatment with trimethylamine in benzene at 60° for a period of four days formed a mixture of L-alpha-(dioleyl) lecithin (VII) and oleyl lysolecithin that could be readily separated by chromatography on silicic acid.

The L-alpha-lecithin thus obtained is an almost colorless, wax-like material whose analytical values for carbon, hydrogen, nitrogen, and phosphorus agreed closely with those required by theory for the lecithin structure shown by formula VII. The unsaturated lecithin was found to be readily soluble at room temperature in methanol, ethanol, or chloroform and, in contrast to the fully saturated lecithins, it was also freely soluble in ether or 90% acetone. The considerable solubility of dioleyl lecithin (and presumably of other unsaturated phosphatides) in moist acetone indicates that the conventional procedures for the isolation of phosphatides from tissues, egg yolk, and other natural sources permit loss of considerable amounts of the more unsaturated phosphatides. This has been noted by members of our department who have been interested in the determination of tissue lipids. In the case of liver for instance the loss can amount to as much as 40% of the organic phosphorus

REACTION SCHEME V



soluble in fat solvents. The specific rotation of $+6.2^{\circ}$ and molecular rotation of $+49^{\circ}$ of L-alpha-(dioleyl) lecithin were identical not only with those of Hanahan's unsaturated dipalmitoleyl lecithin but also with the corresponding values reported by us for the saturated L-alpha-distearoyl lecithin.

The dioleyl lecithin, like Hanahan's dipalmitoleyl lecithin, is quite stable towards atmospheric oxidation. A sample exposed in a thin layer to air for a period of five days had neither become colored nor had it changed its iodine value. It appears, therefore, that the rapid deterioration of unsaturated lecithins obtained from natural sources is not a property of the *pure* unsaturated lecithins.

The catalytic reduction of L-alpha-(dioleyl) lecithin in acetic acid with platinum as catalyst gave in excellent yield optically pure L-alpha-(distearoyl) lecithin. The synthesis of unsaturated lecithins by our method hence proceeds without racemization; a point that had to be proved. The D- or the DL-alpha-(dioleyl) lecithins can be obtained by the same procedure but using the L- or DL-acetone glycerols as starting materials.

The infrared spectrum of the dioleyl lecithin resembles closely that of the saturated lecithins. Although the fully saturated lecithins possess also a strong absorption band at 10.33 μ , the occurrence of this band in the infrared spectrum of the unsaturated lecithin called for caution since compounds with trans-double bonds show a strong absorption at the same wave length. Hence the possibility that elaidinization of the oleic acid had occurred had to be checked. The unsaturated fatty acid obtained by saponification of the synthetic phosphatide, was found, however, to be pure oleic acid.

The availability of a pure unsaturated lecithin made it possible to study the anomalous solubilities of certain substances in the presence of lecithins. It has been the experience of most workers who have tried to isolate natural phosphatides by extraction with ether, chloroform, or alcohol, that the phospholipid extracts contain appreciable amounts of inorganic salts, carbohydrates, amino acids, and other substances that normally would be insoluble in these solvents. Several years ago we had made a qualitative study of the solubilizing effect of ether or chloroform solutions of saturated lecithins or cephalins on sodium chloride, sodium sulphate, glucose, and glycogen. The observed increases in solubility, especially in ether, were, however, rather small, mainly because of the very low solubility of the saturated lecithins

TABLE V

Approximate values (mgm.) of solubility of sodium chloride, L-serine, glucose, and sucrose in 100 ml. of a 5% solution of L-alpha-(dioleyl) lecithin in dry ether, moist ether, or chloroform at room temperature (25°)

	Anhydrous ether	Moist ether	Chloroform
Sodium chloride	0	25	0
L-Serine	0	15	0
Glucose	125	315	175
Sucrose	15	150	115

TABLE VI

CONFIGURATION AND STRUCTURE OF GLYCEROLPHOSPHATIDES ISOLATED FROM BIOLOGICAL SOURCES

L-α-	Glycerylphosphorylethanolamine	Ox brain: L-cr-	Plasmalogen Phosphatidyl serine	yolk phos- From beef brain: L-a- Frem' ox brain: L-a-	above: L-or-		
Glycerophosphoric acid: L-a-	Glycerylphos	Beef brain: L-α-	Cephalins	Distearoyl cephalin From a hydrogenated mixture of egg yolk phosphatides: L-α-	Partially unsaturated cephalin of egg yolk Parent compound of distearoyl cephalin above: L-α-		
	Glycerylphosphorylcholine	Beef pancreas autolyzates: L-α-	Lecithins	Disterryl lecithin From a hydrogenated mixture of egg yolk or brain phosphatides: L- α -	Partially unsaturated lecithin of egg yolk or brain Parent compound of distearoyl lecithin above: L.q.	Dipalmitoyl lecithin Cistiercus fasciolaris: L-a-	Dipalmitoleyl lecithin

in this medium. These studies were now continued by us, but on a more quantitative basis and with the use of the much more ether-soluble L-alpha-(dioleyl) lecithin. The materials to be tested, that is sodium chloride, glucose, sucrose, and serine, were shaken with 5% solutions of the unsaturated lecithin in ether or chloroform at 25° for one hour. The amount of the dissolved material was determined by weighing the remaining solid. Although our investigation was rather limited in scope, its results which are shown on Table V establish definitely the solubilizing effect of lecithins. Of the four substances tested, glucose and sucrose were found to be soluble to an appreciable amount in lecithin solutions. For instance, in a 5% solution of the unsaturated lecithin in moist ether the weight of the dissolved glucose amounted to 6% of the weight of the lecithin. Although the effect on serine seems rather small it is quite possible that other amino acids could be more soluble. The solubilizing effect of lecithins has also been the cause of considerable difficulties in the purification of our synthetic dioleyl lecithin which tenaciously retained barium salts.

In conclusion, I would like to draw your attention to the frequency with which the alpha-structure and L-configuration occurs in natural glycerolphosphatides. Indeed the results of all of our investigations concerning the structure and configuration of natural glycerolphosphatides suggest strongly that these compounds occur in nature only as L-alpha-isomers. In order to show more clearly the uniform structural and configurational pattern of the naturally occurring glycerolphosphatides that has emerged from our investigations, the pertinent data have been collected and are shown on Table VI. It is noteworthy that the natural glycerolphosphatides, and their phosphorus-containing intermediates thus far investigated, were found without exception to be derivatives of L-alpha-glycerophosphoric acid. The occurrence of glycerolphosphatides in nature apparently in only one of two possible enantiomeric forms would suggest a common principle or pathway at some point of their synthesis. Our findings seem to justify a prediction made by Dr. Hermann Fischer and myself almost 20 years ago which stated that the glycerolphosphatides of higher plants and animals would be found to possess the alpha-structure and L-configuration.

References

1. Baer, E. and Maurukas, J. J. Biol. Chem. 212: 25. 1955.
2. Becker, G., Bode, F., and Schrade, W. Klin. Wochschr. 31: 593. 1953.
3. Bode, F. and Ludwig, U. M. Klin. Wochschr. 32: 1097. 1954.
4. Hanahan, D. J. and Jayko, M. E. J. Am. Chem. Soc. 74: 5070. 1952.
5. Lesuk, A. and Anderson, R. J. J. Biol. Chem. 139: 457. 1941.
6. Schrade, W., Becker, G., and Bohle, E. Klin. Wochschr. 32: 27. 1954.
7. Sowden, J. C. and Fischer, H. O. L. J. Am. Chem. Soc. 63: 3244. 1941.
8. Thannhauser, S. J., Benotti, J., and Boncoddo, N. F. J. Biol. Chem. 166: 669. 1946.

DISCUSSION: C. S. McArthur¹

I should like to compliment Dr. Baer and his associates on the elegant syntheses which they have developed for many of the naturally occurring phosphoglycerides. Few biochemists

¹Contribution from the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan.

would care to make this attack on the synthetic flank, and yet progress in the investigation of the biochemistry of the lipids would be slow and unsure without an exact knowledge of the structures and chemical reactions of these substances.

Since the work reported by Dr. Baer leaves little chance for differences of opinion, I shall confine my remarks to a few questions.

N. H. Tattrie and I (4) have described a simple procedure for the preparation of large amounts of crystalline L- α -glycerylphosphorylcholine and could we acylate this ester completely many products of biological interest would be available. However, Baer and Kates (1) reported unsuccessful attempts to carry out this reaction with acyl halides in the presence of pyridine. Similarly, Hanahan (2) found that he could not prepare lecithin from hysoiccithin using acyl halides or acid anhydrides. At first we thought that esterification of the second hydroxyl group might be hindered through cyclic orthoester formation but this explanation seems no longer valid since Baer and Buchnea have shown that the L- α -glycerylphosphoric have shown that the L- α -glycerylphoric have shown that the L- α -glycerylphoric have shown that the L- α -glycerylphoric have shown that the L- α -g ester of ethylene chlorohydrin may be completely acylated by an acyl chloride in the presence of pyridine. One must conclude that it is the influence of the quaternary ammonium group which prevents acylation from going to completion. In this connection I would like to know whether acyl azides may be used in the acylation of hydroxyl groups, and if so, would it be worthwhile attempting the direct synthesis of lecithins from L-α-glycerylphosphorylcholine with such reagents.

In a recent paper, Hanahan, Rodbell, and Turner (3) reported that snake venom lecithinase A specifically removes the fatty acid moiety from the α -position regardless of the degree of saturation of the acid. Has the effect of asymmetry on the action of this enzyme been studied by comparing the results obtained with L- α -lecithins and p- α -lecithins?

Dr. Baer has mentioned the extraordinary solubility of the synthetic lecithins containing unsaturated fatty acids. I should like to ask if he thinks the usual extraction sequence, using acetone to dehydrate the tissue prior to extraction of the phospholipids with alcoholic solvents, might lead to serious losses of the lecithins if the lipids contained in the first aqueous acetone extractions are not recovered?

BABE, E. and KATES, M. J. Am. Chem. Soc. 72: 942. 1950.
 HANAHAN, D. J. J. Biol. Chem. 207: 879. 1954.
 HANAHAN, D. J., RODBELL, M., and TURNER, L. D. J. Biol. Chem. 206: 431. 19.
 TATTRIE, N. H. and McArthur, C. S. Can. J. Biochem. Physiol. 33: 761. 1955.

GENERAL DISCUSSION

Dr. E. Baer .- May I reply to your second question first, since it may be dealt with readily. As far as I am aware no work concerning an investigation of the stereospecificity of the release of the α -fatty acid from lecithins by lecithinase A has been published. The D- α -lecithins which would be required are obtainable only by synthesis. Since this is an involved procedure, I suppose if any investigation had been carried out I would have received a request for the D-isomer of the α -lecithin.

I am at a loss to give a chemical reason why the acylation of glycerylphosphorylcholine stops at the lysolecithin stage. Your suggestion that it is the quaternary ammonium group which interferes with the acylation going to completion is a very interesting one and seems to be borne out by our observation that the CdCl₂ addition compound of glycerylphosphorylcholine, in which the base is more or less inactivated, can be readily acylated to give the

CdCl₂ addition compound of lecithin.

With regard to your last question, whether there may be a significant loss of phosphatides when tissues are dried with acetone prior to extraction of phospholipids with alcohol and ether, I would say emphatically—yes. However, I would prefer that Dr. C. C. Lucas of our department answer your question since he has recently investigated this problem in consider-

able detail and has available some rather striking data.

Dr. C. C. Lucas.—In preliminary experiments on the phospholipid content of a number of tissues from rabbits, Dr. Ridout and I have found that a very large proportion of the total phospholipid phosphorus is extracted along with water and glycerides by acetone. The acetone extracts are taken to dryness in vacuo and the residue treated with petroleum ether-chloroform mixtures, and it is the phosphorus soluble in such mixtures which is referred to as phospholipid phosphorus. Much more work will have to be done on this material before we would be prepared to publish the results but we are reasonably certain that the material actually is phospholipid.

THE CHEMISTRY OF THE PHOSPHOINOSITIDES¹

By J. Folch and F. N. LeBaron

Abstract

The name of inositol phosphatides or phosphoinositides is given to a rather heterogeneous group of substances having in common the presence of inositol, phosphoric acid, and fatty acid(s) among their constituents. The first evidence of their natural occurrence was obtained by Anderson, in 1930, who showed their presence in the lipides of the tubercle bacillus. Since then they have been found in many animal and vegetable tissues, and some of them have been isolated as relatively pure compounds. The majority, if not all of them, are acidic substances which, when prepared by the use of neutral solvents, are obtained as monophosphates of inorganic bases. The constituent inositol is present as a monoester of phosphoric acid, except in the case of brain diphosphoinositide, where it occurs as inositol metadiphosphate. With one possible exception, phosphoinositides contain 1 mole of glycerol per mole of inositol; less commonly, they contain carbohydrates and amines. While valuable information has been obtained on certain details of their chemical structure, in no case has the identification of a phosphoinositide been completed.

Introduction

The name of phosphoinositides or inositol-phosphatides is given to a rather heterogeneous group of substances having in common the presence of inositol, phosphoric acid, and fatty acid(s) among their constituents. Since in no case has the structure of any of these substances been completely established, we will make no attempt at formulating a nomenclature. Instead we will use the names given by different authors to the preparations they have studied and follow the terminology suggested by Folch and Sperry (14) which gives the name of phosphoinositides to all the members of the group, and otherwise divides them into monophosphoinositides and diphosphoinositides depending on whether the molar ratio of inositol to P is 1:1 or 1:2.

The first evidence of the occurrence of phosphoinositides in nature was obtained by Anderson (1), who, in 1930, showed inositol to be a constituent of a phosphatide from tubercle bacillus. This was part of his extensive work on lipides of acid-fast bacteria, and its continuation led later to the identification of manninositose phosphate (3), in 1938, and of glycerol inositol diphosphoric acid (29), in 1947, possible inositol-containing "building blocks" of tubercle bacillus phosphatides. In the meantime, Klenk and Sakai (17), in 1939, isolated inositol monophosphate among the products of hydrolysis of soybean phosphatides, and Folch and Woolley (15), in 1942, identified inositol as a constituent of a brain cephalin fraction. Since then, research in the field has become increasingly active. It has resulted in the isolation of phosphoinositide preparations which meet some limited criteria of purity, namely, soybean "lipositol" by Woolley (30), in 1943, brain "diphosphoinositide" by Folch (8), in 1946, "glycerinositophosphatide" from groundnut

¹Manuscript received December 1, 1955. Contribution from the McLean Hospital, Waverley, Mass., and the Harvard Medical School, Boston, Mass. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955. lecithin by Malkin and Poole (23), in 1953, and "glyceroinositophosphatidic acids" from wheat germ and from heart muscle by Faure and Morelec-Coulon (5, 6), in 1953 and 1954. In addition, phosphoinositides have been studied in corn by Carter et al. (4) and Scholfield et al. (27), in linseed by McGuire and Earle (19), in soybean by a number of workers (9, 11, 16, 17, 24, 25, 26, 27, 28), in fish by Lovern and Olley (18), in liver by Macpherson and Lucas (21), and McKibbin (20), and in brain by Hawthorne and Chargaff (16), and Folch and LeBaron (12, 13).

From all this work a large body of information has been accumulated and, as is often the case in very active fields, the evidence presented ranges all the way from well established facts to simple speculation. In such a situation the reviewer must choose between being comprehensive and exercising the right of selection. Comprehensiveness may well result in a lengthy, confusing, and necessarily uncritical exposition of many insufficiently substantiated claims along with well established facts. The alternative of selecting the important material puts a heavy burden on the reviewer, in the sense that, in many cases, information upon which to base a selection is simply not available, because much of the work published is acknowledgedly in the nature of preliminary or exploratory studies. Faced with this dilemma these writers have decided to present the subject matter by reviewing, in the first place, the well established facts, proceeding, then, through the information available in descending order of certainty. To that effect we will divide our subject matter into three different sections, namely, a first one which will deal with the simple constituents of phosphoinositides, a second one which will discuss the structures resulting from the combination of said constituents, and a third one in which the different phosphoinositides will be described.

Constituents of Phosphoinositides

The following substances have been isolated among the products of terminal hydrolysis of phosphoinositides: inositol, phosphoric acid, fatty acids, glycerol, amines, tartaric acid, and sugars.

Inositol

Of the nine possible stereoisomers of inositol, the one that has been found in all phosphoinositides in which its isomeric form has been determined, is *i*- or *meso*-inositol. This is not surprising, since *meso*-inositol, of all the isomers, is by far the most commonly found in living tissue. In keeping with this point, it should be ascertained whether or not scyllitol takes the place of *meso*-inositol in phosphoinositides from plagiostomes, since their tissues contain scyllitol in abundance.

Fatty Acids

Crude fatty acid fractions have been obtained and roughly quantitated in preparations from liver (20), brain (10, 12, 13), linseed oil (19), and tubercle bacilli (2, 29). More careful work has been done on the fatty acids from the

phosphoinositide of soybean (25, 30), groundnut oil (23), wheat germ (5), and cardiac muscle (6). Woolley (30) studied the fatty acids of soybean lipositol by separating the petroleum ether-soluble material from a hydrolyzate into saturated and unsaturated fatty acid fractions by the lead soap method. He showed that the unsaturated fraction was all oleic acid by determining its iodine number and hydrogenating it to stearic acid. The saturated acids were separated as magnesium salts and a small amount (5%) of cerebronic acid was identified. The rest of the mixture had the elementary composition and melting point of a mixture of 70% palmitic acid and 30% stearic acid. The total saturated acids were present in an amount equimolar to the total unsaturated acids. It seems possible that the cerebronic acid came from an Okuhara (25) has succeeded in isolating myristic acid from his soybean phosphoinositide preparation. Malkin and Poole (23) studied the fatty acids of groundnut phosphoinositide by hydrogenation and crystallization of the mixture of methyl fatty esters and potassium soaps obtained by its alkaline hydrolysis. They concluded that the fatty acids were a mixture of 47% oleic, 23% linoleic, and 17% palmitic acids. Faure and Morelec-Coulon (5) found that the fatty acids in the inositol phosphatidic acids of wheat germ were constituted by an approximately equimolar mixture of palmitic and C₁₈ unsaturated acids. They found, in contrast (6), that the fatty acids of cardiac muscle preparations were an approximately equimolar mixture of stearic acid and an unsaturated acid mixture with an iodine value of 220.

Glycerol

Although it might appear natural that inositol would take the place of glycerol in phosphatides, the fact is that glycerol has been found to be a constituent of all phosphoinositides in which it has been looked for by adequate methods. Thus, glycerol has been isolated and/or estimated quantitatively in the glycerinositophosphatidic acids of wheat germ (5), and cardiac muscle (6), in the soybean monophosphoinositide isolated by Folch (9, 11), and in brain diphosphoinositide (10); in all of which it has been found to be present in equimolar ratio with inositol. In the case of groundnut phosphoinositides (23), and tubercle bacillus phosphatides (2), the presence of glycerol has been established by the isolation of glycerophosphoric acid. In summary, the only exception to this general rule would be lipositol. However, in the case of lipositol, no attempt was made to estimate glycerol. Also, a water soluble, alcohol soluble phosphate ester fraction, with many of the properties of glycerophosphoric acid was obtained among the products of partial hydrolysis and this was claimed to be inositol monophosphate on the strength of the barium content of its barium salt. In the precipitation of barium glycerophosphate a mixture of glycerophosphate and acid glycerophosphate is often obtained, with the result that the barium content of the precipitate is low. Therefore it is conceivable that the phosphate ester fraction referred to above may, in fact, have been glycerophosphoric acid.

Sugars

Some phosphoinositides appear to contain carbohydrates, although in many cases the sugars reported to be present in inositol-rich phosphatide preparations may be impurities. Water soluble carbohydrate-containing substances, as well as free inositol itself, are usually present in solvent extracts of tissue lipides unless or until the tissue or extracts are *exhaustively* washed or dialyzed. Unfortunately much of the work reported here on phosphoinositides was done with unwashed or partially washed material. Cerebroside contamination, of course, presents a different and more difficult problem.

Thus far three sugars, mannose, galactose, and arabinose, have been found in hydrolyzates from phosphoinositide preparations. Mannose was isolated from tubercle bacillus phosphatides by Anderson's group (2, 3, 29), and, although their preparations were rather crude and undialyzed, they isolated a polysaccharide containing inositol and mannose from partial hydrolyzates, so it appears that mannose and inositol are combined and presumably in the phosphatide. The isolation and identification of this substance will be discussed later.

Arabinose and galactose have been found in dialyzed, purified, phosphoinositides from soybean (11, 16, 24, 30) and groundnut (23). The sugars from soybean have been identified chromatographically. In the case of groundnut phosphoinositides, Malkin and Poole identified the two sugars chromatographically on paper and also isolated them chromatographically and identified them by physical characteristics and derivatives. As is true of the tubercle bacillus phosphoinositides, larger fragments have been isolated from soybean and groundnut which contain both inositol and sugars. These will also be discussed later.

Amines

In many cases the phosphoinositides are apparently free of nitrogen, and, in fact, lack of nitrogen has been used as a criterion of purity. However, in some cases nitrogen bases are apparently actual constituents of the phosphoinositide molecule.

Ethanolamine is one of the constituents found by Malkin and Poole (23) in their groundnut oil preparation and they have identified it in hydrolyzates, both chromatographically and by isolation and preparation of the flavianate. The only nitrogen bases present were ethanolamine and ethanolamine phosphate. This was also true of the complex ester they isolated, so it would appear that the ethanolamine is bound to the inositide molecule. Ethanolamine was also found in soybean lipositol preparations by Woolley (30) but the amounts reported (0.44%) were so small that the possibility of its being present only as a contaminant in the undialyzed preparation could not be eliminated. Folch (11) also reported an amine in soybean monophosphoinositide in approximately the molar proportions of 1:2 with inositol, but the actual substance was not identified.

Carter and co-workers (4) have isolated a sphingosine-type base from a purified phosphatide fraction of corn lipides which contained 7-11% inositol.

This base, which is the same as cerebrin base previously found in yeasts and molds, has been named phytosphingosine. We assume Dr. Carter will discuss it more comprehensively in his review. The phytosphingosine isolated from corn phosphatide accounted for about five per cent of the weight of the purified phosphatide and 20-25% of its nitrogen. While the source was a highly purified inositol phosphatide fraction, it cannot yet be conclusively stated that phytosphingosine is a constituent of a phosphoinositide molecule.

A sphingosine-like base has also been reported to be present in phosphatidopeptides, a new type of phosphoinositides from brain (13), but the actual identity has not yet been established. A second type of amino compound occurs in phosphatido-peptides, also, since at least 15 different amino acids have been found chemically bound to the phosphoinositide as polypeptide chains. The amino acids in hydrolyzates have been separated and determined by starch column chromatography. The fact that they are combined was shown by α -amino nitrogen determinations before and after hydrolysis. When first isolated these phosphatido-peptides were obtained from material which had been subjected to trypsin digestion, but similar preparations have also been obtained without enzymatic digestion and they also contained inositol phosphate and combined amino acids linked chemically.

Tartaric Acid

t

ì

e

d

t

g

t

Tartaric acid has only been isolated from lipositol (30). This finding will be discussed in relation to lipositol itself.

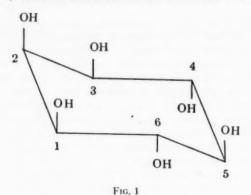
Products of Partial Hydrolysis of Phosphoinositides

Inositol Phosphates

All the evidence available indicates that inositol is present in all phosphoinositides as a phosphate ester. This has been established in the case of the different preparations we are discussing, either by actual isolation of inositol monophosphate, chromatographic identification, or, in the case of brain diphosphoinositide by the isolation of inositol metadiphosphate.

The chemical structure of inositol metadiphosphate, isolated among the products of hydrolysis of diphosphoinositide, has been established (10) by: (a) elementary analysis, the results of which closely agreed with theoretical values calculated from the postulated formula; (b) isolation from it of inositol in theoretical yield; (c) titration to pH 8.2, which showed that each phosphoryl radical in the molecule had two free acid groups; and (d) the study of the products of its reaction with periodic acid. It was found that 2 moles (M.) of HIO4 were used, and 1 M. of formic acid was produced per mole of inositol diphosphate. One mole of inositol p-diphosphate would have used 2 M. of HIO4 without any production of formic acid, while 1 M. of inositol o-diphosphate would have used 3 M. of HIO4 with production of 2 M. of formic acid.

No stereochemical studies have been made on inositol phosphates and it would seem advantageous to undertake them. Magasanik and Chargaff (22)



have suggested that the actual stereochemical structure of *meso*-inositol is the chair form (Fig. 1) with the largest possible number of equatorial hydroxyl groups. In this case the hydroxyl on position 2 is the only polar one. A priori, it would seem that a single phosphoryl would be least hindered sterically if it were substituted on the inositol molecule in the polar 2 position. By the same criterion the diphosphate would most easily be esterified at either the 2,4- or the 2,6-positions. Polarimetric studies would be valuable in this case since the 2,4- and 2,6-disubstituted inositols would be optically active.

Manninositose Phosphate

Anderson, Lothrop, and Creighton (3) have isolated a phosphorylated mannose inositol glycoside from the phosphatides of acid-fast bacteria. Their purified phosphatide fraction was saponified with a slight excess of alcoholic potassium hydroxide at room temperature, and a water soluble organic phosphate was obtained, the elementary composition of which corresponded roughly to that for mannose inositol diphosphoric acid. Upon hydrolysis with dilute ammonia at 170° for 8.5 hr., phosphorus was quantitatively removed leaving a polysaccharide which had no reducing power. When this saccharide was hydrolyzed by boiling in dilute sulphuric acid, mannose and inositol were released in the approximate molecular ratio of 2:1. The evidence was interpreted by the authors as indicating that mannose was tied through its reducing group to inositol, and that there was no indication of the location of the two phosphoryl groups.

Inositol Glycerol Diphosphoric Acid

The phosphatides of acid-fast bacteria were also the source of another complex phosphate ester isolated by de Sütö-Nagy and Anderson (29). This was obtained after hydrolysis by boiling 1% alcoholic potassium hydroxide for two hours, followed by fractionation of the lead salts of the organic phosphates liberated. Simple phosphates were precipitated with neutral lead acetate and then the more complex one with basic lead acetate. The elementary composition of the complex ester thus obtained was C₂H₁₆O₁₄P₂Ba₂.

When this ester was subjected to strong acid hydrolysis (10% sulphuric acid at 160° for three and one-quarter hours) glycerol and inositol were obtained "in approximately theoretical amounts". After milder hydrolysis (10% sulphuric acid at 45° for six hours) the barium salts of inositol monophosphate and glycerophosphate were isolated. Thus it appeared that the original complex ester was a combined diphosphate of inositol and glycerol. However, since the P: Ba ratio was 1:1, both phosphoryl radicals were of necessity monosubstituted, i.e. they were terminal groups. Therefore glycerol and inositol could not be combined through a phosphate bridge.

Phosphoinositides

As stated in the introduction, there are five different preparations of phosphoinositides which have been prepared and studied in such a way that they may be considered to be "pure" substances in the limited sense that they meet one or more criteria of homogeneity, such as having all of their P and/or N as one single radical, remaining of constant composition through a reasonable number of procedures of fractionation, etc. These relatively pure preparations are soybean lipositol, brain diphosphoinositide, groundnut glycerinositophosphatide, and wheat germ and cardiac muscle glyceroinositophosphatidic acids. Another one will also be referred to, namely, a soybean monophosphoinositide which is different from lipositol and which has a somewhat weaker claim to "purity".

General Properties

Not much information has been given by the respective authors on the properties of phosphoinositides. However, it appears that most of them are difficult to dissolve in dry solvents and are more easily soluble in solvents saturated with water. They are all acidic phosphatides, and, in the cases in which it has been studied quantitatively, when they are obtained by the use of neutral solvents they are monophosphates, i.e. they contain one equivalent of inorganic base per mole of phosphorus.

Contaminants

Before describing each individual phosphoinositide it might be pertinent to discuss the question of contaminants, a problem only too well known to lipide chemists. The most likely contaminants in any phosphatide preparations are either other lipides, mainly other phosphatides, or non-lipide substances. The presence or absence of contaminanting lipides can be established both by analysis for likely contaminants and by exhaustive procedures of fractionation through which a presumably pure phosphoinositide should remain of constant composition. The problem of non-lipide contaminants may vary in complexity according to the natural source of the particular preparation being studied. All phosphoinositides contain dialyzable, water soluble contaminants which should be removed either by exhaustive dialysis, by repeated precipitation of the phosphatide from an aqueous emulsion, or by

partition between adequate biphasic systems. The problem of undialyzable or water insoluble non-lipide contaminants is a more troublesome one which can not be solved by any general treatment. In fact this is a problem that places a question mark after many observations in phosphatide chemistry, and which should always be kept in mind in the interpretation of results. For instance, any substance present in phosphatide hydrolyzates either in very small amounts, or in amounts not bearing a simple numerical relation to the main constituents, should be considered to be a contaminant, in the absence of positive proof to the contrary.

Lipositol

The first phosphoinositide with any claim to "purity" was lipositol which was isolated from soybean by Woolley in 1942 (30). It was obtained from commercial soybean lecithin by an elaboration of the method of Folch (7) for fractionating brain cephalin. The resulting preparation was an acidic phosphatide, easily emulsifiable in water, and soluble only in wet organic solvents. By analysis, and by study of the products of partial or complete hydrolysis, the following compounds were identified as possible constituents: inositol, phosphoric acid, galactose, oleic acid, and saturated fatty acids (v.s.), which were found to be present in essentially equimolar concentrations; and ethanolamine and tartaric acid, which were found at lesser concentrations. Although no structure was postulated by the author, some clues about it were obtained by the isolation of inositol monophosphate and, possibly, of ethanolamine tartrate and inositol galactoside tartrate. However, the small amount of ethanolamine isolated weakens any claim to its being a constituent.

By comparison with other phosphoinositides, lipositol is notable for the absence of glycerol and the presence of tartaric acid among its constituents. However, as was discussed in a preceding section, the claimed absence of glycerol appears to be open to reasonable doubt. As for the presence of tartaric acid, the possibility of its being an impurity cannot be dismissed, because the method of preparation of lipositol did not include dialysis or any other step intended to remove water soluble contaminants, an omission which was especially unfortunate in the case of soybean phosphatides which are rich in all types of carbohydrate contaminants. This reservation must also be applied to the finding of ethanolamine tartrate and the galactoside. In summary, the absence of glycerol and the presence of tartaric acid and ethanolamine among the constituents of lipositol are still open questions.

Brain Diphosphoinositide

Diphosphoinositide is an acidic phosphatide which was isolated from brain cephalin by Folch in 1946 (8, 10). Neutral solvents were employed in its preparation and it was obtained as a salt of calcium and magnesium with the base: phosphorus molar ratio of a monophosphate. The potassium salt was prepared by treating the above preparation with potassium oxalate, and it was obtained as a microcrystalline powder. The constituents of diphosphoinositide are fatty acids, inositol metadiphosphate (v.s.), and glycerol in

Fig. 2.

equimolar proportions. A small varying amount of nitrogen was found which is felt by the author to be a contaminant.

The structure of diphosphoinositide is not completely established, but the available evidence indicates that part of the molecule is represented by Fig. 2 in which R and R' stand for unknown radicals. Since monoglycerides have been isolated among the products of mild acid treatment of diphosphoinositide, one of these radicals may well be a monoglyceride. This would leave one radical unaccounted for. One possibility is that diphosphoinositide is, in fact, a polymer of the basic unit, inositol diphosphate – glycerol – fatty acid, and that the vacant valency on one of the phosphoryl groups would be involved in the linkage between monomers. The fact that the potassium salt of diphosphoinositide is truly soluble in water and undialyzable would argue in favor of such a large molecule structure.

Glycerinositophosphatide of Groundnut

Malkin and Poole (23) have isolated from groundnut a phosphoinositide containing glycerophosphoric acid, inositol monophosphate, and ethanolamine in equimolar proportions, with three sugar molecules and two fatty acids to each glycerol. They subjected their purified and partially dialyzed phosphoinositide preparation to mild alkaline hydrolysis at room temperature by treating a moist benzene solution of it with one-third volume of methanolic potassium hydroxide (9.6 gm. KOH to 120 ml. MeOH) for 20 hr. The insoluble phosphates released were fractionated by the consecutive use of netural and basic lead acetate according to the method of de Sütö-Nagy and Anderson (29). A complex ester, soluble in neutral lead acetate and insoluble in basic lead acetate, was obtained which had the composition shown in Table I.

This ester was chromatographed on paper with three different solvent mixtures: (a) n-butanol saturated with water; (b) n-butanol saturated with water: morpholine, 3:1; and (c) pyridine saturated with water: ethyl acetate: water, 4.5:10:10. Each chromatogram was run for 60 hr. and

TABLE I

Composition of complex ester from groundnut phosphoinositide

Constituent	Groundnut ester	Formula 3
Carbon (%)	36.5	36.7
Hydrogen (%)	5.8	5.8
Nitrogen (%)	1.46	1.58
Phosphorus (%)	6.8	7.0
Inositol (Scherer Test)	+	+
Sugars (Molisch Test)	+	+
Glycerol	+	+
Reducing groups (Fehling's Test)		_
Free amino groups (Ninhydrin Test)	_	
Neutral equivalent (Phenolphthalein)	445.4	441.5

sprayed with ammoniacal silver nitrate for sugars or with perchloric acid—molybdate reagent for phosphates. In all cases the papers exhibited only one spot which contained both the phosphates and sugars. From this it was concluded that there was only one substance present. The following compounds were demonstrated chromatographically in hydrolyzates of this substance: galactose, arabinose, ethanolamine, ethanolamine phosphate, inositol, glycerol, inositol monophosphate, and α - and β -glycerophosphates. The latter three phosphates were isolated and separated as barium salts in the usual way, and, by the weights of the phosphates isolated, the ratio of glycerol to inositol was estimated to be 1:1.04. The sugars were isolated chromatographically and estimated quantitatively by periodate oxidation, giving a molecular ratio of galactose: arabinose of 1:1.89. By mild acid hydrolysis of the whole phosphoinositide a disaccharide of arabinose and galactose had previously been isolated.

The water solution of this complex ester was neutral to methyl red although it could be titrated to determine a neutral equivalent with phenolphthalein. The authors therefore concluded that it had no strong acid equivalent and both phosphate groups were in "the same state of weak acidity".

On the strength of the evidence gathered, the authors suggested for the complex ester either one of the two structures given in Fig. 3. One alternative to Fig. 3a is that the sugars be joined to inositol. This alternative would leave the amino group free, which conflicts with the negative ninhydrin reaction shown by the ester. A stronger objection to this top structure is that Malkin and Poole have failed to obtain any inositol diphosphate among the products of hydrolysis. Therefore, in the opinion of these reviewers, the structure in Fig. 3b is more likely to be the correct one.

Glyceroinositophosphatidic Acids

Faure and Morelec-Coulon (5, 6) have isolated and analyzed an apparently pure phosphoinositide. They succeeded in crystallizing substances from wheat germ and cardiac muscle which had compositions consistent with the

(a)

Fig. 3.

но но

OH

HO

(9)

Fig. 4.

formula they suggested (Fig. 4). In both cases the substances were obtained by precipitation with ethanol from an ether solution of the acetone insoluble "phosphatidic acids" which had been extracted from the tissue by methanol. These precipitates were redissolved and purified as barium salts, the final crystallization being from methanol. The final steps had to be varied from wheat germ to cardiac muscle because of the differing nature of the impurities present. Table II shows the comparative analyses.

No partial hydrolysis studies have yet been made on these crystals to isolate larger fragments, such as organic phosphates, so the structure in Fig. 4 is only one of a number of possibilities. The authors make the statement that periodate oxidation of the intact phosphatidic acid completely destroyed the inositol while leaving a substantial part of the glycerol intact. It is difficult to interpret this statement without knowing what criteria were used

TABLE II

Composition of crystalline glyceroinositophosphatidic acids

Constituents	Wheat germ	Formula 4	Cardiac muscle
Na salt			
Phosphorus (%)	3.56	3.61	3.38
Nitrogen (%)	0.09) Before	0	< 0.03
Glucose (%)	0.29 crystal-	0	< 0.05
Unsaponifiable matter (%)	0.6 lization	0	0
Iodine No.	56	59	84
Fatty acids	Equimolar palmitic and unsat. C ₁₈	Equimolar palmitic and linoleic	Equimolar stearing and mixture of unsat. acids with I2 No. of 220
Ba salt			
Molecular ratio, P/Ba	2.0	2	1.95
Free acid Molecular ratios			
Fatty acids/P	2.1	2	2.03
Glycerol/P	0.96	1	0.97
Inositol/glycerol	1.05	1	1.05

to follow inositol destruction. Simple loss of inositol per se means only that two adjacent hydroxyl groups were available for oxidation, but, if the oxidation products were studied and the inositol was completely oxidized, it could show that the inositol was, in fact, bound at one point.

Soybean Monophosphoinositide

A monophosphoinositide preparation has been isolated from soybean which is apparently different from lipositol. It was first isolated by Folch (9, 11) who found it to be an acidic phosphatide and to contain a primary amine, phosphoric acid, inositol, glycerol, a monosaccharide, and fatty acids in the molar ratios 1:2:2:2:2:3. No products of partial hydrolysis were isolated, and the only evidence for the possible homogeneity of the material was the observation that it could not be fractionated further by a number of different procedures. Therefore the reviewers feel that its claim to being considered a "pure" phosphoinositide is a weak one.

Similar preparations of undetermined degree of purity have been obtained and studied by two other groups of workers. Hawthorne and Chargaff (16) obtained chromatographic evidence for the presence of arabinosides and galactosides of inositol monophosphate among the products of hydrolysis of their preparation. Okuhara and Nakayama (25) methylated their intact purified phosphatide and subsequently isolated a small amount of material whose properties were consistent with those of pentamethyl inositol. They therefore concluded that the inositol in the intact phosphatide was monosubstituted.

In the way of concluding remarks, we would like to re-emphasize that much of the information reported here is based on possibly adequate, but sketchily presented evidence. Some of today's "facts" may well be disproved by later work. By the same token, many observations that appear today to be no more than learned guesses may well be the opening leads to fruitful lines of work.

References

1. Anderson, R. J. J. Am. Chem. Soc. 52: 1607. 1930.

2. Anderson, R. J. Yale J. Biol. and Med. 15: 311. 1942-43.

- 3. Anderson, R. J., Lothrop, W. C., and Creighton, M. M. J. Biol. Chem. 125: 299. 1938.
- CARTER, H. E., CELMER, W. D., LANDS, W. E. M., MUELLER, K. L., and TOMIZAWA, H. H. J. Biol. Chem. 206: 613. 1954.
- FAURE, M. and MORELEC-COULON, J. Compt. rend. 236: 1104. 1953.
 FAURE, M. and MORELEC-COULON, J. Compt. rend. 238: 411. 1954.
- FOLCH, J. J. Biol. Chem. 146: 35. 1942.
 FOLCH, J. Federation Proc. 5: 134. 1946.
- 9. Folch, J. Federation Proc. 6: 252. 1947. 10. Folch, J. J. Biol. Chem. 177: 497, 505. 1949.
- 11. Folch, J. In McElroy, W. D. and Glass, B. Phosphorus metabolism. Vol. II.
- The Johns Hopkins Press, Baltimore, Md. 1952.

 12. FOLCH, J. and LEBARON, F. N. Federation Proc. 10: 183. 1951.

 13. FOLCH, J. and LEBARON, F. N. Federation Proc. 12: 203. 1953.

- FOLCH, J. and SPERRY, W. M. Ann. Rev. Biochem. 17: 147. 1948.
 FOLCH, J. and WOOLLEY, D. W. J. Biol. Chem. 142: 963. 1942.
- 16. HAWTHORNE, J. N. and CHARGAFF, E. J. Biol. Chem. 206: 27.
- 17. KLENK, E. and SAKAI, R. Z. physiol. Chem. 258: 33. 1939.
- 18. LOVERN, J. A. and OLLEY, J. Biochem. J. 55: 686. 1953.
- 19. McGuire, T. A. and Earle, F. R. J. Am. Oil Chemists' Soc. 28: 328. 1951.
- 20. McKibbin, J. M. Federation Proc. 13: 262. 1954.
- 21. MACPHERSON, L. B. and LUCAS, C. C. Federation Proc. 6: 273. 1947.
- 22. MAGASANIK, B. and CHARGAFF, E. J. Biol. Chem. 174: 173. 1948.
- 23. MALKIN, T. and POOLE, A. G. J. Chem. Soc. 3470. 1953.
- 24. Nomura, D. J. Japan. Chemistry, 3: 145. 1949. Chem. Abstr. 46: 3962c. 1952.
- 25. OKUHARA, E. and NAKAYAMA, T. J. Biol. Chem. 215: 295. 1955.
- Scholfield, C. R. and Dutton, H. J. J. Biol. Chem. 208: 461. 1954.
 Scholfield, C. R., Dutton, H. J., Tanner, F. W., Jr., and Cowan, J. C. J. Am. Oil Chemists' Soc. 25: 368. 1948.
- SCHOLFIELD, C. R., McGuire, T. A., and Dutton, H. J. J. Am. Oil Chemists' Soc. 27: 352. 1950.
- 29. DE SÜTÖ-NAGY, G. I. and ANDERSON, R. J. J. Biol. Chem. 171: 749, 761. 1947.
- 30. WOOLLEY, D. W. J. Biol. Chem. 147: 581. 1943.

DISCUSSION: C. C. Lucas¹

I should like to congratulate Dr. Folch on his excellent presentation of a most interesting group of compounds. Although it is over 100 years since Scherer (7) first isolated inositol from muscle and named it "muscle sugar", relatively little is known even yet as to its role in metabolism. It occurs widely in nature, sometimes free, more often combined, and in both water-soluble and fat-soluble forms. Thanks to the studies of Anderson, Klenk, Folch, and others in recent years more is known about the forms in which it occurs. Inositol has been found in biological materials as diverse as pine sawdust and tubercle bacilli; in the juice of sugar beets and the latex of rubber trees as well as in heart, skeletal muscle, brain, soya beans, and peanuts. Its ubiquitous occurrence suggests that it is possibly an essential cellular component.

In 1928 Miss E. V. Eastcott (3), working in Professor Lash Miller's laboratory, in Toronto, succeeded in isolating Bios I and showed it to be *meso*-inositol. This demonstration that inositol is an essential nutrient for yeast was followed eventually by proof that it is also required by fungi and certain molds. Bacteria do not seem to require it; they can produce it from simpler carbon compounds. In 1940 Woolley (9) showed that inositol is required by mice, and since then certain other mammals have been found to require it, at least under some dietary conditions.

While it is now known that the formation of ethers, esters, glucosides, and lipids containing inositol occurs in plants and animals, in no case is the site or mechanism of these

synthetic activities known.

R. H. Smith (8) has recently shown the presence of inositol in the hydrolyzate of phosphatides isolated from rubber latex. Here, as in many other cases where inositol has been found in a phospholipid, reducing sugars were also liberated during the hydrolysis. Galactose. glucose, and an unidentified ketose were present. It may be pertinent to recall that about two years ago Brown and Serro (2) isolated a new type of inositol complex from the juice of sugar beets which proved to be the galactoside of meso-inositol. About a year ago Hawthorne and Chargaff (5) isolated an inositide from soybean phosphatides and presented chromato-graphic evidence which led them to believe that it might be the phosphoric ester of such an inositol galactoside. They also believed that they had evidence for the presence of the phosphoric ester of an arabinoside of inositol.

In 1941, Gavin and McHenry (4) reported that inositol exerts a lipotropic effect in rats, an observation which was confirmed in several laboratories, including ours. My own interest in the inositol-containing phosphatides dates from about 1943. Dr. Beveridge and I (1) considered the possibility that inositol exerts its lipotropic effect through some phosphatide combination. With Lloyd B. Macpherson (6) we began a study of the inositol-containing phosphatides in rat liver; later we turned our attention to the more available pig liver and soybean phosphatides as sources of the substances in question. I do not intend to make any detailed comments on these studies. We never did succeed in isolating a fraction that we considered was near a pure compound, but Macpherson obtained fractions greatly enriched

¹ Contribution from the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario.

in inositol. Much time and material were expended in attempts to improve upon the microbiological assay for inositol. Use of the so-called inositol-less Neurospora mutant was very time-We felt it was somewhat more accurate than the yeast method but it requires consuming. a considerably longer time—almost a week before an answer was available. The yeast method had a considerable element of uncertainty—we never felt that the results of any particular assay could be relied upon within about ± 10 –15%. Moreover, some inositol linkages were fairly resistant to hydrolysis and the rather severe conditions necessary to liberate the inositol caused significant destruction of the portion set free.

Dr. Macpherson and I were particularly intrigued by the basic fraction of the hydrolyzate obtained from the inositol-rich fraction from liver phospholipids. As most of you know, Dr. Macpherson has gone to Dalhousie University and since his departure, work on this subject

has ceased in our laboratory.

I wonder if Dr. Folch would mention the conditions of hydrolysis and method of assay he now finds most useful in his work on these compounds, and whether he would care to make any further comments on the nature of the basic material in any of the phosphoinositides with which he has worked.

BEVERIDGE, J. M. R. and LUCAS, C. C. J. Biol. Chem. 157: 311. 1945. BROWN, R. J. and SERRO, R. F. J. Am. Chem. Soc. 75: 1040. 1953. EASTCOTT, E. V. J. Phys. Chem. 32: 1094. 1928. GAVIN, G. and MCHENRY, E. W. J. Biol. Chem. 139: 485. 1941. HAWTHORNE, J. N. and CHARGAFF, E. J. Biol. Chem. 206: 27. 1954. MACPHERSON, L. B. and LUCAS, C. C. Federation Proc. 6: 273. 1947. SCHERER, D. Ann. 73: 322. 1850. SMITH, R. H. Biochem. J. 57: 130. 1954; 57: 140. 1954; 56: 240. 1954. WOOLLEY, D. W. Science, 92: 384. 1940. J. Biol. Chem. 136: 113. 1940.

GENERAL DISCUSSION

Dr. J. Folch.—We heat the sample in a sealed tube with 6 N hydrochloric acid for 40 hr. at 109°C. and use the yeast method (Saccharomyces cereviseae) as modified by McKibbin. This modification takes care of the interfering effect of choline in the hydrolyzate. Destruction of inositol is negligible. The uncertainty of the assay appears to be of the order cited by Dr. Lucas.

With respect to nitrogenous bases, it must be kept in mind that some phosphoinositides are apparently free from nitrogen, although others definitely do contain a basic constituent. Ethanolamine has been identified in peanut phosphoinositide, phytosphingosine in the corn product, and amino acids in polypeptide chains appear to be bound in some phosphoinositides

No new data on "unidentified bases" are available.

CHEMISTRY OF THE SPHINGOLIPIDES1

By H. E. CARTER, DEMETRIUS S. GALANOS,² and Y. FUJINO³

Abstract

Chemical studies leading to the elucidation of the structures of sphingosine, cerebrosides, sphingomyelin, and cerebroside sulphuric acid are The general nature of the complex sphingolipides (ganglioside, hematoside, globoside, strandin, polycerebroside) is discussed. Preliminary studies on the nature of the phytosphingosine-containing lipides of corn phosphatides are presented.

Introduction

In the last few years considerable progress has been made in studies of the chemistry of the sphingolipides. The structure of sphingosine has been completely elucidated and sphingosine and dihydrosphingosine have been synthesized. The structures of the cerebrosides and sphingomyelins have been established although their synthesis has not yet been accomplished. A number of more complicated derivatives of sphingosine-gangliosides, hematoside, globoside, strandin, polycerebroside-have been described and await precise chemical characterization. The presence in plant phosphatides of a long-chain base-phytosphingosine-has been reported and its structural relationship to sphingosine determined.

It is my purpose to review certain of these areas with the goal of giving you a quick look at the advances and certain of the unsolved problems in the sphingolipide field.

Sphingosine

Several early workers contributed to knowledge of the structure of sphingosine following its discovery by Thudichum. Levine and West (30) and Klenk (19) established that sphingosine was a dihydroxyaminooctadecene-4. A particularly valuable contribution to these studies was the report of Klenk (19) on the preparation of triacetylsphingosine from the mixture of crude bases obtained on hydrolysis of cerebrosides. Triacetylsphingosine crystallizes beautifully from the pyridine - acetic anhydride reaction mixture. It is easily purified and stable over long periods of time. It has, therefore, been of unusual value in the preparation and characterization of sphingosine.

Carter and co-workers (6), using the periodate reagent on dihydrosphingosine, determined the relative position of the functional groups and established the structure of sphingosine as 1,3-dihydroxy-2-aminooctadecene-4.

¹Manuscript received December 1, 1955.

Contribution from the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana, Ill. This investigation was supported in part by a research grant (No. B-574) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955.

Visiting Scientist in Chemistry, University of Illinois, under the auspices of the International Cooperation Administration, United States Government.

Present address, Obihiro University, Obihiro, Hokkaido, Japan.

The stereochemistry of sphingosine has been determined in the last two or three years. The D configuration was originally assigned to carbon 2 by Carter and Humiston (8) and this conclusion has been confirmed by Kiss, Fodor, and Banfi (16) and more recently by Klenk and Faillard (23).

The question as to whether the third carbon bears an *erythro* or *threo* relationship to the second has been attacked in several ways. In our laboratories the synthesis and characterization \mathbf{p} -*erythro*- and \mathbf{p} -*threo*- α -amino- β -hydroxystearic acid made possible the ready synthesis of the *erythro* and *threo* forms of dihydrosphinogsine (10).

Comparison of the synthetic bases with the natural isomer was complicated by the racemic nature of the synthetic materials. However, this problem was solved by the observation that D-erythro-dihydrosphingosine gave an excellent yield of a tribenzoyl derivative closely similar in properties to the derivative of the natural base, whereas under identical conditions the D-threo base failed completely to yield a tribenzovl derivative. Subsequent resolution of the D-erythro base yielded an isomer identical in all its properties with the natural base. Thus it was established conclusively that the sphingosine isolated through the triacetyl derivative by the procedure of Klenk has the erythro configuration. Jenny and Grob (15) reached the same conclusion on the basis of synthetic studies involving a stereospecific synthesis of the erythro base from trans-octadecenoic acid. Kiss et al. (16) also reached the same conclusion using a completely different approach. These workers obtained by the ozonolysis of triacetylsphingosine a four-carbon fragment which was characterized by comparison with known derivatives of erythro-α-amino- β , γ -dihydroxybutyric acid.

These results provide a direct proof of both the D configuration of the amino carbon and the *erythro* configuration of the asymmetric system in sphingosine (and dihydrosphingosine).

The *trans* structure of the double bond in sphingosine was shown by the infrared studies of Mislow (34) and of Marinetti and Stotz (33). These data together establish finally the complete structure of sphingosine as D-erythro-1,3-dihydroxy-2-amino-4-*trans*-octadecene.

The synthesis of sphingosine has been reported recently (39) and the procedure seems capable of being developed into a practical process. In view of the difficulties of obtaining pure sphingosine by hydrolysis of cerebrosides the synthetic procedure should be highly valuable to workers in this field.

These results do not prove, however, that sphingosine as it exists in cerebrosides actually has the erythro configuration. The difficulty arises from the fact that hydrolysis of N-acyl derivatives of 1,2-amino-alcohols by acidic reagents may result in inversion of configuration. The yield of sphingosine by the Klenk procedure is so poor that the three isomer could well be present and indeed has been isolated by Seydel by aqueous methanolic hydrolysis of cerebrosides (38, see also reference 15). Furthermore Carter et al. (9) obtained both the three and the erythre O-methyl ethers of sphingosine by the methanolic sulphuric acid hydrolysis of cerebrosides. In order to avoid this difficulty Fujino, in our Laboratories, undertook to develop a procedure for the hydrolysis of cerebrosides which would eliminate the possibility of inversion during hydrolysis and which would give a good yield of a single isomer of sphingosine or dihydrosphingosine. For these studies, pure phrenosine and dihydrophrenosine were prepared in quantity. A preliminary study was made of the hydrolysis of dihydrophrenosine by anhydrous methanolic acids in the hope that removal of the allylic system would minimize or eliminate inversion and O-methyl ether formation. Only partial success was achieved. No O-methyl ethers were formed and the yield of erythro-dihydrosphingosine (50%) was considerably higher than by the usual procedures. However, a significant quantity of the three isomer might have been present in the hydrolysis mixture (although none could be isolated).

Attention was next directed to the alkaline hydrolysis of phrenosine. By a modification of Klenk's barium hydroxide procedure (18), psychosine (galactosidosphingosine) was obtained in excellent yield. (It is of interest that dihydrophrenosine is hydrolyzed by barium hydroxide much more slowly than is phrenosine.) Catalytic reduction gave dihydropsychosine in quantitative yield. Hydrolysis of dihydropsychosine with aqueous-ethanolic hydrogen chloride gave an excellent yield of *erythro*-dihydrosphingosine characterized as the tribenzoyl derivative (m.p. 145–146°) and the triacetyl derivative (m.p. 100–102°). These reactions are shown in the following equations.

Since only the *erythro* isomer was obtained and in excellent yields by a process which would not be expected to lead to inversion, it seems certain that sphingosine as it exists in the phrenosine molecule has the *erythro* configuration. Similar studies should perhaps be carried out on sphingomyelin although it seems highly probable that only one isomer of sphingosine occurs in the various natural sphingolipides.

The Structure of Cerebrosides

Early workers had shown that cerebrosides contain a fatty acid attached to the amino group in an amide linkage and galactose bound glycosidically to one of the hydroxyls of sphingosine. Carter and Greenwood (7) established the point of attachment by determining the behavior of hexaacetylphrenosine on catalytic reduction over an active platinum oxide catalyst. Under these conditions triacetylsphingosine undergoes hydrogenolysis of the allylic acetoxyl group yielding acetic acid and sphingine (8). Hexaacetylphrenosine also yielded acetic acid in about the same amount and no galactose derivative was liberated. Hydrolysis of the hydrogenolysis products gave sphingine.

$$CH_{3}(CH_{2})_{12}CH=CH-CH-CH-CH-CH_{2}$$

$$O \quad NH \quad O \quad NH \quad O$$

$$Ac \quad CO \quad CH_{2}$$

$$CHOAc \quad H-C-OAc \quad CHOAc \quad H-C-OAc \quad CH_{2}$$

$$(CH_{2})_{21} \quad AcO-C-H \quad O \quad (CH_{2})_{21} \quad AcO-C-H$$

$$CH_{3} \quad AcO-C-H \quad CH_{3} \quad AcO-C-H$$

$$H-C_{2} \quad CH_{2}OAc \quad CH_{2}OAc$$

Hexaacetylphrenosine

These results establish that the galactose moiety of phrenosine (and by analogy other cerebrosides) is attached at the first carbon of sphingosine and that phrenosine has the following structure. Nakayama (35) has reached similar conclusions using a different approach.

Phrenosine (erythro)

Attempts to determine the configuration of the galactosidic bond have given contradictory results. Hamasato (14) found that psychosine sulphate was hydrolyzed by β -galactosidase but not by α . On the other hand, Kiss and Jurcsik (17) observed hydrolysis of cerebrosides by an α -galactosidase and presented other evidence for the α -galactosidic linkage. This problem is the one unsolved question concerning the structure of cerebrosides.

The Structure of Cerebroside Sulphuric Ester

A cerebroside sulphuric ester was first isolated from beef brain in 1933 by Blix (1). It contained cerebronic acid, sphingosine, galactose, and sulphuric acid. Recently, Thannhauser and co-workers (40, 41) have obtained this material in a purified form and have shown by methylation studies that the sulphuric acid is attached at the 6 carbon atom of galactose as shown in the following formula:

The Structure of Sphingomyelin

It has been established that sphingomyelins consist of ceramides attached to phosphorus and choline in some way. The isolation of phosphorylcholine and sphingosine phosphate as hydrolysis products (36, 37) of sphingomyelin establishes that the choline is attached through a phosphate ester bond to one of the hydroxyl groups of sphingomyelin. Fujino (12) has presented evidence based on methylation studies that the phosphorylcholine moiety is attached to the primary alcohol group of sphingosine. More recently, Stotz and co-workers have presented conclusive proof for the correctness of this structure by two independent procedures (32, 37). In the first approach periodate oxidation of sphingosine phosphate (obtained by alkaline hydrolysis of sphingomyelin) was shown to yield glycolaldehyde phosphate. This result would be given only by the structure shown below:

In the second approach sphingomyelin was hydroxylated by performic acid and the resulting product was oxidized by periodate. From the cleavage product serine was obtained as shown in the following equations. These results carry somewhat more weight since the degradation was performed on intact sphingomyelin which had not been subjected to vigorous alkaline treatment.

m

pl

NH₂

Sphingomyelin therefore has the structure shown above.

It is interesting to note that sphingomyelin on acid hydrolysis gives a mixture of sphingosinephosphorylcholine, sphingosine phosphate, and phosphorylcholine, each of the phosphate ester bonds undergoing some cleavage. In the alkaline hydrolysis also both sphingosine and sphingosine phosphate were produced.

Complex Glycosides of Sphingosine

Probably the first indication of the existence of substances analogous to the cerebrosides but containing carbohydrate moieties other than or in addition to galactose came from investigations of the lipides accumulating in spleen and brain in the various lipoidoses (Gaucher's disease, Tay-Sachs disease, etc.). The presence of glucose rather than galactose in the kerasin-like cerebroside which accumulates in the spleen of a Gaucher patient was reported by Halliday and co-workers (13) in 1940. The presence of an aminohydroxy acid (neuraminic acid) in a cerebroside-like material accumulating in the brain of a Tay-Sachs patient was observed by Klenk (20) in 1935 and subsequently the term ganglioside was applied to those cerebroside-like lipides containing neuraminic acid (28). Gangliosides and glucose-containing cerebrosides have since been found to be components of normal tissue.

In 1951 Yamakawa and Suzuki (44) isolated from the stroma of horse erythrocytes a complex lipide to which the term hematoside was given. Hydrolysis of hematoside in methanolic acid yielded fatty acid, sphingosine, galactose (2 moles), and a water-soluble aminohydroxy acid, hematiminic acid, which was isolated as a methoxyl derivative. The parent carbohydrate was designated prehematiminic acid. These observations were confirmed by Klenk and co-workers (24, 29) who established that prehemataminic acid was identical with neuraminic acid and hemataminic acid with the methoxyl derivative of neuraminic acid, the substance obtained on hydrolysis of gangliosides in anhydrous methanolic acid. Further studies by Yamakawa and by Klenk of the red cell stroma lipides of other species (human, cow, sheep, goat, hog, dog, chicken) have turned up evidence for a complex and confusing array of different sphingolipides.

Before discussing these substances it is necessary to say a few words about neuraminic acid and its relationship to sialic acid and hexosamine. Neuraminic acid was originally detected in lipides as a substance giving a typical deep red color on heating with Bial's reagent (orcinol-hydrochloric acid) and giving marked humin formation with aqueous acids. A substance with similar properties—sialic acid—had been discovered earlier by Blix (2) as a component of submaxillary mucin. Sialic acid analyzed for C₁₄H₂₃O₁₁N and was considered to be an unusual disaccharide containing acetylhexosamine, a polyhydroxy acid (not hexuronic), and a second acetyl group. Subsequently Blix and co-workers (3) isolated galactosamine from gangliosides and submaxillary mucin. It seemed possible at that time that the hexosamine present in gangliosides constituted part of the neuraminic (or perhaps sialic acid) moiety. More recent evidence tends to refute this view although the problem

is not solved and it seems hardly worth while to review the details until more definitive data on the structure of neuraminic and sialic acid are available. Suffice it to say that the methoxyl derivative of neuraminic acid does not give a test for hexosamine nor yield hexosamine on acid hydrolysis, while gangliosides (from brain) do give a strong positive reaction for hexosamine (21). On this basis, Klenk believes that neuraminic acid and hexosamine are separate components of gangliosides. Furthermore, Blix et al. (4) have indicated in a subsequent publication that galactosamine in gangliosides and mucin is loosely bound to sialic acid and not part of it.

Klenk and Faillard (22) have recently isolated N-acetylneuraminic acid ($C_{12}H_{21}O_{10}N$) from mucin by mild aqueous degradation. This substance yields the methoxyl derivative of neuraminic acid on treatment with acidic methanol. It also appears to be closely related chemically to sialic acid (the empirical formula differs only by the presence of a second acetyl group in sialic acid). The N-acetyl derivative has not, however, been obtained from gangliosides. Some definitive structural studies on neuraminic and sialic acids are essential as a basis for a satisfactory characterization of the gangliosides and related substances.

Based on the assumption that hexosamine and neuraminic acid are separate entities, Klenk and Yamakawa have found some interesting species differences in the lipides of the red cell stroma (26, 43, 47). The blood cell of the horse and dog are predominantly of the hematoside type and contain neuraminic acid, but not much hexosamine. On the other hand, the erythrocyte lipides of the human, sheep, goat, and hog are of the "globoside" type (45), and contain hexosamine but no neuraminic acid. Bovine erythrocytes possess both; chicken, neither (46). Recently, Klenk and Lauenstein (25) found that glucosamine rather than galactosamine was present in the sphingolipides of bovine erythrocytes. These differences may be quantitative rather than qualitative in view of the results of Klenk's careful fractionation of horse erythrocyte lipides, which disclosed the presence of a second fraction containing both neuraminic acid and hexosamine and still a third substance with no nitrogenous component but with more than one hexose residue per molecule.

To this array of complicated glycolipides should be added strandin (11) from brain (hexosamine but no neuraminic acid) and "polycerebroside" from Gaucher spleen (42) which has neither hexosamine nor neuraminic acid.

The chemical characterization of these complex substances presents many problems whose interest is enhanced by the suggestions that these lipides may be related to the immunological properties of red blood cells (22, 43).

The Sphingolipides of Plants

Since the discovery of inositol in soybean phosphatides (27) several studies of the plant phosphatides have appeared. It is not my intention to review this literature since I am primarily concerned with one particular component

of the plant phosphatides. In 1954 Carter and co-workers (5) reported the presence of a long-chain base in corn phosphatides. The structure of this base was established as shown below:

In view of the close structural similarity to sphingosine the term phytosphingosine was assigned to this substance.

The discovery of this base in plant phosphatides raises some interesting biochemical questions and leaves several as yet unsolved structural problems. The stereochemistry of the asymmetric centers, the nature of the closely similar unsaturated base in soybean phosphatides, the nature of the lipides in which phytosphingosine occurs are all interesting problems. Currently, most of our attention is being devoted to attempts to isolate homogeneous phytosphingolipides from corn phosphatides. A countercurrent system (butanol, methanol, water, heptane) has afforded promising results and given evidence for the presence of at least two fractions containing phytosphingosine. This work has not progressed far enough to merit further discussion but I would like to mention briefly an attempt we have made to determine whether phytosphingosine is attached directly to carbohydrate in a cerebroside-like molecule or whether it exists mainly as phosphate ester derivatives. For this purpose an extension of the "sphingosine" determination of McKibbin and Taylor (31) was devised. In the McKibbin-Taylor procedure lipide samples are hydrolyzed first with barium hydroxide and then with hydrochloric acid. This two-step procedure presumably converts both sphingomyelins and cerebrosides to sphingosine. The hydrochloride salt of the base then is extracted from the acidic solution by chloroform. The chloroform-soluble nitrogen is taken as a measure of long-chain base since low molecular weight amines remain in the aqueous hydrochloric acid layer.

The first step in this procedure (barium hydroxide hydrolysis) cleaves amide linkages and may partially degrade the phosphate-choline bond in sphingomyelin. Under these conditions glycosidic bonds are not cleaved and cerebrosides are converted to psychosine. It seemed likely that if the barium hydroxide hydrolyzate were acidified and extracted at this point only the sphingosine phosphate derivatives would be extracted, leaving psychosine hydrochloride in the aqueous layer. A control study with known phrenosine substantiated this prediction, thereby making possible a modified procedure with some promise as a method of distinguishing between "cerebroside" and "sphingomyelin" types of sphingolipides.

Application of the two procedures to soybean and corn phosphatides gave different "sphingosine" values with alkaline hydrolysis alone and with alkaline followed by acid hydrolysis. These results strongly suggest that both sphingomyelin and cerebroside types are present in these phosphatide mixtures, a conclusion which is in agreement with the results of our fractionation studies.

In conclusion, I would like to point out that the development of a synthetic procedure for sphingosine and determination of the structures of the cerebrosides and sphingomyelins begins to establish a solid chemical basis for what I am sure are going to be very interesting biochemical studies of the metabolism and function of the sphingolipides in the years ahead.

References

1. BLIX, A. Z. physiol. Chem. 219: 82. 1933. 2. BLIX, G. Z. physiol. Chem. 240: 43. 1936.

3. BLIX, G., SVENNERHOLM, L., and WERNER, I. Acta Chem. Scand. 4:717. 1950.

4. BLIX, G., SVENNERHOLM, L., and WERNER, I. Acta Chem. Scand. 4: 717. 1950.

4. BLIX, G., SVENNERHOLM, L., and WERNER, I. Acta Chem. Scand. 6: 358. 1952.

5. CARTER, H. E., CELMER, W. D., LANDS, W. E. M., MUELLER, K. L., and TOMIZAWA, H. H. J. Biol. Chem. 206: 613. 1954.

6. CARTER, H. E., GLICK, F. J., NORRIS, W. P., and PHILLIPS, G. E. J. Biol. Chem. 142: 449. 1942; 170: 285. 1947.

449. 1942; 170: 265. 1947.

7. CARTER, H. E. and GRERNWOOD, F. L. J. Biol. Chem. 199: 283. 1952.

8. CARTER, H. E. and HUMISTON, C. G. J. Biol. Chem. 191: 727. 1951.

9. CARTER, H. E., NALBANDOV, O., and TAVORMINA, P. A. J. Biol. Chem. 192: 197. 1951.

10. CARTER, H. E., SHAPIRO, D., and HARRISON, J. B. J. Am. Chem. Soc. 75: 1007. 1953.

11. FOLCH, J., ARSOVE, S., and MEATH, J. A. J. Biol. Chem. 191: 819. 1951.

12. FUJINO, Y. J. Biochem. (Japan), 39: 45. 1952.

13. HALLIDAY, N., DEUEL, H. J., JR., TRAGERMAN, C. J., and WARD, W. E. J. Biol. Chem. 132: 171. 1940.

132:171. 1940.

 НАМАБАТО, Y. Tôhoku J. Exptl. Med. 53: 35. 1950.
 JENNY, E. F. and GROB, C. A. Helv. Chim. Acta, 36: 1936. 1953. KISS, J., FODOR, G., and BANFI, D. Helv. Chim. Acta, 37: 1471.
 KISS, J. and JURCSIK, I. Acta Chim. Acad. Sci. Hung. 5: 477.
 KLENK, E. Z. physiol. Chem. 153: 74. 1926.
 KLENK, E. Z. Physiol. Chem. 153: 74. 1926. 1054

19. Klenk, E. Z. physiol. Chem. 185: 169. 1929 20. Klenk, E. Z. physiol. Chem. 235: 24. 1935. 1929.

KLENK, E. Z. physiol. Chem. 288: 216. 1951.
 KLENK, E. and FAILLARD, H. Z. physiol. Chem. 298: 230. 1954.
 KLENK, E. and FAILLARD, H. Z. physiol. Chem. 299: 48. 1955.

KLENK, E. and LAUENSTEIN, K. Z. physiol. Chem. 288: 220. 1951.
 KLENK, E. and LAUENSTEIN, K. Z. physiol. Chem. 291: 249. 1952.
 KLENK, E. and LAUENSTEIN, K. Z. physiol. Chem. 295: 164. 1953.

KLENK, E. and SAKAI, R. Z. physiol. Chem. 258: 104.
 KLENK, E. and SAKAI, R. Z. physiol. Chem. 258: 33. 1939.
 KLENK, E. and SCHUMANN, E. Ber. 75B: 1632. 1942.
 KLENK, E. and WOLTER, H. Z. physiol. Chem. 291: 259. 1952.
 LEVENE, P. A. and WEST, C. J. J. Biol. Chem. 16: 549. 1913-14; 18: 481. 1914.
 MCKIBBIN, J. M. and TAYLOR, W. E. J. Biol. Chem. 178: 29. 1949.
 MARINETTI, G., BERRY, J. F., ROUSER, G., and STOTZ, E. J. Am. Chem. Soc. 75: 313. 1053.

1953.

33. MARINETTI, G. and Stotz, E. J. Am. Chem. Soc. 76: 1347. 1954.

MISLOW, K. J. Am. Chem. Soc. 74: 5155. 1953.
 NAKAYAMA, T. J. Biochem. (Japan), 37: 309. 195
 RENNKAMP, F. Z. physiol. Chem. 284: 215. 1949.

37. ROUSER, G., BERRY, J. F., MARINETTI, G., and STOTZ, E. J. Am. Chem. Soc. 75: 310. 1953.

DEL, P. V. Zur Kenntnis des Sphingosine. Dissertation, Eidgenössische Technische Hochschule in Zürich, Zurich, 1941. See also Grob, C. A. and Jenny, E. F. Helv. 38. SEYDEL, P. V. 1952. Chim. Acta, 35: 2106. 1952. 39. Shapiro, D. and Segal, K. J. Am. Chem. Soc. 76: 5894. 1954.

SHAPIRO, D. and SEGAL, K. J. Am. Chem. Soc. 76: 5894. 1954.
 THANNHAUSER, S. J. and BONCODDO, N. Federation Proc. 12: 280. 1953.
 THANNHAUSER, S. J., FELLIG, J., and SCHMIDT, G. J. Biol. Chem. 215: 211. 1955.
 UZMAN, L. L. Arch. Pathol. 55: 181. 1953.
 YAMAKAWA, T. and IIDA, T. Japan. J. Exptl. Med. 23: 327. 1953.
 YAMAKAWA, T. and SUZUKI, S. J. Biochem. (Japan), 38: 199. 1951; 39: 175. 1952.
 YAMAKAWA, T. and SUZUKI, S. J. Biochem. (Japan), 39: 393. 1952.
 YAMAKAWA, T. and SUZUKI, S. J. Biochem. (Japan), 40: 7. 1953.
 YAMAKAWA, T. SUZUKI, S., and HATTORI, T. J. Biochem. (Japan), 40: 611. 1953.

DISCUSSION: J. F. BERRY1

Dr. Carter's excellent and comprehensive review has suggested to us the direction of future research in sphingolipid chemistry. Indeed, the chemical tools which he has presented will probably form the basis for the next developments.

Of particular interest to us will be the advances in the chemistry of lipid complexes such

as the proteolipids and the lipopeptides.

Folch (9) has described the isolation of three water-insoluble proteolipid fractions from brain white matter. These fractions contain lipid and protein in different proportions. The lipid moiety of these complexes appears to be composed largely of cerebrosides or cerebroside

and sphingomyelin.

Folch and LeBaron (8) have also reported the isolation from brain white matter of a phosphorus-containing, trypsin-resistant fraction called phosphatidopeptide or lipopeptide. Schrade and co-workers (2, 14) and Macheboeuf (3) have found similar fractions in serum, plasma, and red blood cells. As many as 13 different amino acids have been identified in the peptide moiety. The brain lipopeptide is extracted with a chloroform - methanol - hydro-chloric acid mixture. The phospholipid moiety from the brain lipopeptide is reported to be

composed of inositol diphosphate, fatty acids, and sphingosine or a sphingosine-like substance.

In this laboratory, cat or rat brain slices incubated with inorganic P22 have been found to yield a P22 labelled fraction which remains after carrier phosphate – trichloroacetic acid extraction, alcohol-ether extraction, and Davidson salt extraction of nucleic acids. prolonged alkaline hydrolysis of this residue, seven radioactive and partially identified phosphorus-containing components may be separated by filter paper electrophoresis. If, however, the chloroform - methanol - hydrochloric acid extraction of Folch is inserted following the alcohol-ether extraction, all but two of these components are removed, suggesting that this non-nucleotide, protein-bound phosphorus may be lipopeptide of the type reported by Folch (8). It is hoped that the proposed hydrolysis studies of Dr. Baer on his synthetic phosphatidopeptide might aid us in identifying this fraction.

Two problems which arise are: (1) whether the lipopeptides are related chemically or metabolically to the proteolipids, and (2) the exact nature of the sphingosine derivatives. The latter problem is presumably one which might be approached by the methods outlined by Dr. Carter. In addition, Wittenberg (15) has recently reported a method of separation

of sphingosine and related compounds by reversed phase partition chromatography.

One problem which, to some extent, has hampered the elucidation of phospholipid structures has been the difficulty encountered in isolation, purification, and determination of individual phospholipids. This problem may become particularly pertinent in dealing with some of the newer sphingolipids and sphingolipid complexes.

Often it has been possible to deal with amounts of tissue sufficiently large that appreciable

losses could be tolerated for the sake of purity.

In many cases, methods of determination have developed which, after solvent extraction, depend on differential hydrolysis or identification and determination of the hydrolysis products. For example, the procedure used by Dawson (5) employs paper chromatographic separation of the hydrolysis products from a total lipid extract.

Such methods often do not take into account the possibility of one hydrolysis product arising common to several lipids some of which may not have been previously described or studied. Brante (4), for example, has reported an unidentified non-choline-containing sphingophospholipid which occurs in the myelin sheath of peripheral nerve.

Recently, attempts to separate intact phospholipids on columns or filter paper have become more numerous (1, 6, 7, 10, 11, 12). Rouser, Marinetti, and Berry (13) have reported successful separations of pure phospholipids on filter paper using a variety of solvents which include combinations of lutidine, acetic acid, chloroform, octanol-2, or methanol. Marinetti and Witter have applied these solvents to the separation of phospholipids from tissue extracts of rats injected with P^{n} . In dealing with these radioactive lipids, it was possible to carry out separations on as little as 5 γ of lipid. My colleagues, Drs. Witter and Marinetti, may wish to comment further on the details and results of their more recent experiments.

In this laboratory, this same procedure has been applied to the separation of lipids from brain slices or homogenates incubated with C¹⁴-glycine, C¹⁴-acetate, or P²⁸.

The usual procedure involves washing of the tissue with trichloroacetic acid and carrier This is followed by extraction with alcohol-ether or chloroform-methanol. The extract is evaporated to dryness under nitrogen and taken up in isoamyl alcohol - benzene. The material is applied in this medium to acid- and methanol-washed Whatman No. 1 filter paper. Octanol/lutidine/acetic acid is allowed to ascend the paper overnight after which it is dried, washed with water, and dipped in aqueous rhodamine G. Positions of phospholipids are indicated by discovered by the control of th are indicated by fluorescent spots under ultraviolet light and by the positions of radioactivity on an autoradiograph.

¹Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario.

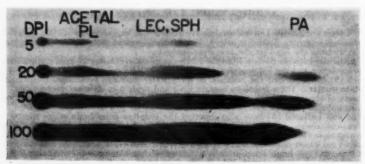


Fig. 1. Ascending octanol/lutidine/acetic acid chromatogram of phospholipid mixture from guinea pig brain slices incubated with P^{zz} . Each spot numbered in μ gm. lipid applied: 5 μ gm., 20 μ gm., 50 μ gm., 100

Abbreviations: DPI, diphosphoinositide; acetal PL, acetal phospholipid; LEC, SPH, lecithin and sphingo-myelin; PA, phosphatidic acid.

A typical autoradiograph from such a chromatogram is shown in Fig. 1. The material was obtained from guinea pig brain slices incubated with Pss. Numbers at the origin used was obtained from guinea pig brain slices incubated with P32. indicate the number of gammas of total lipid applied to each spot.

The spots indicated are diphosphoinositide, acetal phospholipid, lecithin and sphingo-myelin, and phosphatidic acid. By the rhodamine test, a cephalin spot bearing no radio-activity was found between acetal phospholipid and the lecithin-sphingomyelin spot. The composition of the spots was based on the known behavior of pure authentic compounds in this solvent and spot tests. In addition, it was found that a major portion of the radioactivity. this solvent and spot tests. In addition, it was found that a major portion of the radioactivity could be eluted from the paper by five-minute exposure to 5 N HCl at 100°. These hydrolysis products were identified by 2-dimensional chromatography in the solvents of Dawson (5).

Very recently, it was found that 2-dimensional chromatograms of unhydrolyzed lipids could be run. Under these conditions, the diphosphoinositide spot yielded another component, as did the cephalin spot. As yet, no decent separation of lecithin and sphingomyelin has been obtained.

However, it is hoped that these preliminary studies may serve as a basis for a tool which will prove useful in the isolation of phospholipids, the identification of new phospholipids, identification of impurities in phospholipid preparations, and the characterization of sphingolipids appearing in lipid-protein complexes.

- AMELUNG, D. and BÖHM, P. Hoppe-Seyler's Z. physiol. Chem. 298: 199. BECKER, G., BODE, F., and SCHRADE, W. Klin. Wochschr. 31: 593. 1953. BLASS, J., ROUHI, A., LECOMTE, O., and MACHEBOBUF, M. Bull. 80C. chim
- Bull. soc. chim. biol. 35: 959. 1953.
- BRANTE, G. Acta Physiol. Scand. Suppl. 63, 18: 1. 1949.
 DAWSON, R. M. C. Biochim. et Biophys. Acta, 14: 374.

- DAWSON, R. M. C. Biochim. et Biophys. Acta. 14: 374. 1954.
 DIEKERET, J. W. Aund REISER, R. Federation Proc. 14: 202. 1955.
 FILLERUP, D. L. and MEAD, J. F. Proc. Soc. Exptl. Biol. Med. 83: 574. 1953.
 FOLCH, J. and LEBARON, F. N. Federation Proc. 12: 203. 1953.
 FOLCH, J. and LEBS, M. J. Biol. Chem. 191: 819. 1951.
 HECHT, E. and MINK, C. Biochim. et Biophys. Acta. 8: 641. 1952.
 LEA, C. H. and RHODES, D. N. Biochem. J. 57: xxiii. 1954.
 LEA, C. H., RHODES, D. N., and STOLL, R. D. Biochem. J. 60: 353. 1955.
 ROUSER, G., MARINETTI, G., and BERRY, J. F. Federation Proc. 13: 286. 194.
 SCHRADE, W., BECKER, G., and BOHLE, E. Klin. Wochschr. 32: 27. 1954.
 WITTENBERG, J. B. J. Biol. Chem. 216: 379. 1955.

GENERAL DISCUSSION

Dr. Witter.—In connection with Dr. Berry's paper I would like to report some results Drs. Marinetti, Rouser, and I have recently obtained. The lipids of rat heart, brain, liver, intestine, spleen, and lung were isolated from animals which had been injected with radioactive orthophosphate. The lipids were chromatographed in the solvents described by Rouser, Marinetti, Witter, Berry, and Stotz, and radioautographs were developed. As many as seven phospholipids were detected by this method, and this probably represents a minimal However, none of the tissues including brain contained detectable amounts of phosphatidic acid even though several unidentified trace constituents were found and an excess of lipid was applied to the paper. Dr. Berry's experiments are excellent proof of other

claims that phosphatidic acid is found in brain homogenates or slices. This interesting difference between in vivo and in vitro systems must be taken into account when considering the role of phosphatidic acid in the metabolism of the lipids. Perhaps the presence of this acid in tissue slices and homogenates may be the result of an intensification of a pathway which normally occurs in vivo. Our failure to find even traces of phosphatidic acid in tissue phospholipids heavily labelled with P⁴² casts some doubt on the quantitative significance of this pathway.

Dr. Marinetti.-- I should like to emphasize that evidence for the trans configuration of sphingosine does not unequivocally prove that naturally occurring sphingolipids possess the trans structure. The rather drastic acid hydrolysis used to obtain sphingosine would undoubtedly isomerize any cis form to the trans configuration. Studies on intact sphingolipids isolated under mild conditions would give more decisive information regarding this point. Indeed Dr. Carter's finding that the absorption band near 10.3 µ in cerebroside was essentially removed by reduction with hydrogen is better evidence that this sphingolipid has the *Trans* configuration. Publications from our laboratory have confirmed this finding and moreover have shown by infrared spectral analysis that sphingomyelin and N-lignocerylsphingosine have the *trans* structure. Studies on sphingomyelin were complicated by the finding that the covalent phosphate group occurring in this compound absorbs more strongly than the trans double bond in the 10.3μ region. Thus the band at 10.3μ in dihydrosphingomyelin was only significantly diminished and altered in comparison to sphingomyelin. On the other hand the results with N-lignocerylsphingosine and cerebroside were clear-cut since interference by The band occurring near 10.3 µ in both of these compounds was other groups was absent. completely eliminated after reduction. The results on these three sphingolipids strongly indicate that all sphingolipids have the trans configuration in their sphingosine moiety. Studies with N-lignocerylsphingosine were particularly significant because this lipid was isolated from natural sources under very mild conditions involving only extraction and recrystallization from neutral organic solvents thus minimizing any possibility of isomerization during preparation.

THE BIOLOGICAL SYNTHESIS OF PHOSPHOLIPIDS1

BY EUGENE P. KENNEDY

The problem of the biological synthesis of phospholipids has been for decades the subject of interest and speculation. However, no progress was made in working out the chemical pathways by which phospholipids are synthesized in the living cell until the introduction of the isotope tracer technique. Studies in which isotope tracers have been used to investigate the biosynthesis of phospholipids may be divided into three broad categories.

First, there are experiments in which the isotope, usually Pi32, is injected into the intact animal and the rate of formation or breakdown of phospholipid in various tissues is determined². The literature on this topic is too extensive to be considered in detail here, and the reader is referred to reviews by Artom (2, 3) and by Chaikoff and Zilversmit (16). Such studies on the intact animal have greatly enlarged our knowledge of nearly every aspect of the metabolism and function of phospholipids. Nevertheless, it could scarcely be expected that information on the detailed mechanism of the enzymatic synthesis of phospholipids could be obtained from experiments of this type. As an example of the difficulties encountered in attempts to test possible intermediates in the biosynthesis of phospholipid, the work of Riley (49) using phosphorylcholine-P22 may be cited. This investigator found that within 30 min. after injection nearly all of the radioactivity in the blood was present as inorganic phosphate, and that phosphorylcholine-P32 did not differ significantly from Pi32 as a precursor of labeled phospholipid in various tissues of the rat. Clearly the labeled compound was almost entirely hydrolyzed before it could penetrate to the site of the enzymes carrying out the synthesis of phospholipid.

Zilversmit, Entenman, and Fishler (58) have described the theoretical relationship which should exist between the specific activity of a labeled product and its biological precursor at various times after the administration of a single dose of an isotopic tracer. In applying the formulae derived by Zilversmit *et al.* several simplifying assumptions must be made, as pointed out by these authors. Furthermore, one must be able to isolate the precursor compound in a pure form for accurate determinations of specific activity, which can scarcely be done if in fact the precursor is a novel compound the

Contribution from the Ben May Laboratory for Cancer Research and the Department of Biochemistry, University of Chicago, Chicago, Illinois. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955

¹ Manuscript received December 1, 1955.

²The following abbreviations will be used in this paper: ATP, adenosine triphosphate; UTP, uridine-5'-triphosphate; CTP, cylidine-5'-triphosphate; GDP, guanosine-5'-diphosphate; ITP, inosine triphosphate; Pi, inorganic orthophosphate; PO—P, inorganic pyrophosphate; α -GP, α -glycerophosphate; β -GP, β -glycerophosphate; CDP-choline, cylidine diphosphate choline; CDP-chanolamine, cylidine diphosphate ethanolamine; UDP-choline, uridine diphosphate choline; GDP-choline, guanosine diphosphate choline; ADP-choline, adenosine diphosphate choline.

properties of which cannot be predicted. Such considerations have severely limited the usefulness of attempts to determine the biological precursors of phospholipids by these methods.

In a more direct attack on the problem, Chaikoff and his collaborators (21, 23) studied the uptake of P_i^{32} in the phospholipids of slices of liver and brain. These workers made the important observation that sources of metabolic energy were essential for the observed incorporation, and concluded that there was little or no equilibration of lipid P with P_i^{32} by reactions not requiring the input of free energy via coupled reactions. This conclusion has been amply borne out by later work. The tissue slice technique, however, appears to offer no real advantages over isotope tracer studies with intact animals in identifying the actual intermediates involved in the synthesis of phospholipids.

A third type of application of isotope tracers to the problem involves the use of cell-free enzyme systems. Although earlier attempts (21) to demonstrate the synthesis of phospholipids in suspensions of broken cells were unsuccessful, Friedkin and Lehninger (22) in 1948 showed that cell-free particulate preparations of rat liver vigorously incorporated P_i^{32} into an unidentified lipid fraction. This reaction was found to be completely dependent upon the maintenance of oxidative phosphorylation. It thus became possible to investigate the biosynthesis of phospholipid in cell-free preparations with some reasonable hope of separating the various enzyme systems involved and isolating intermediates in the reaction sequence. The present discussion will deal solely with recent research in this field.

The Enzymatic Synthesis of Phosphatidic Acids

L-\alpha-GP as an Intermediate in the Biosynthesis of Phospholipids

Isotope tracer studies in vivo by Popják and Muir (47) and Zilversmit, Entenman, and Chaikoff (57) led these workers to suggest that α -GP might be a precursor of phospholipids. Proof of such a function of α -GP has been provided by the work of Kennedy (28) and of Kornberg and Pricer (39, 40).

In an investigation of the incorporation of P_i^{32} into the lipids of isolated rat liver mitochondria, Kennedy (28) found in confirmation of the work of Friedkin and Lehninger (22) that the enzymatic generation of radioactive ATP from P_i^{32} was essential for this process (Reaction [1] of Fig. 1). Further, the rate of incorporation was approximately doubled by the addition of glycerol, which suggested that the second reaction in the sequence was the phosphorylation of glycerol to yield α -GP. This postulated role of α -GP was confirmed by three experimental findings. First, synthetic DL- α -GP was converted to a lipid product at a rate exceeding that of the incorporation of P_i^{32} . Secondly, radioactive α -GP enzymatically synthesized from P_i^{32} could be isolated from the mitochondrial reaction mixture. Finally, when unlabeled DL- α -GP was added to the enzyme system under conditions in which P_i^{32} was the sole source of radioactivity, the expected isotope dilution

with consequent diminution of incorporation of radioactivity was noted. β -GP was inactive in this enzyme system, in confirmation of the work of Baer and Kates (4, 5) which indicates that naturally occurring glycerophosphatides are derivatives of α -GP.

$$P_{1}^{22} + ADP + 13,000 \text{ cal.} \xrightarrow{(1)} ATP^{32} + CHOH$$

$$CH_{2}OH$$

$$CH_{2}OH$$

$$CH_{2}OH$$

$$CH_{2}OH$$

$$CH_{2}OH$$

$$CH_{2}OH$$

$$RC-O-CH O \longleftrightarrow (4) HOCH O + RC-SCoA$$

$$CH_{2}O-P^{32}-OH OH$$

$$OH OH$$

$$(3)$$

$$RCOOH + ATP + CoA$$

The Enzymatic Phosphorylation of Glycerol

Although considerable evidence had been brought forward by previous workers (8, 26) suggesting the existence of an enzyme catalyzing the phosphorylation of glycerol (Reaction [2] of Fig. 1), no direct proof of this step had been reported. In 1954, Bublitz and Kennedy (11) isolated and partially purified an enzyme from rat liver which catalyzes the reaction:

Fig. 1

[1] ATP + glycerol
$$\longrightarrow$$
 L- α -GP + ADP.

This enzyme, called glycerokinase, also catalyzes the phosphorylation of two other substrates, dihydroxyacetone and L-glyceraldehyde. UTP or CTP may be substituted for ATP in Reaction [1] but ITP is inactive.

When small amounts of purified glycerokinase are added to isolated mitochondrial enzyme systems, the rate of incorporation of P_i^{32} into phospholipids is approximately doubled (11) showing that the concentration of glycerokinase in the enzyme particles is a rate-limiting factor in the over-all reaction sequence, and further demonstrating that it is $\text{L-}\alpha\text{-GP}$ which is an intermediate in phospholipid synthesis in this system. This finding again confirms the earlier work of Baer (6, 7) from which it could be concluded that the natural glycerophosphatides are of the L-configuration.

According to the conventional point of view, the glycerol molecule appears to be symmetrical, but it should be noted that it is phosphorylated in asymmetrical fashion by this enzyme. Only L- α -GP is formed, whereas if the enzyme could not differentiate between the two apparently identical primary hydroxyl groups of the glycerol molecule, a mixture of D- α -GP and L- α -GP

would result. The asymmetrical metabolism of such apparently symmetrical substances has now become widely recognized since the original hypothesis of Ogston (46) on the role of citric acid in the tricarboxylic acid cycle was verified experimentally (48). Schwartz and Carter (54) have pointed out that glycerol contains a *meso*-carbon atom and have presented a discussion of the stereochemistry of such substances in relationship to purely chemical and to enzymatic reactions.

Schambye, Wood, and Popják (51) and Swick and Nakao (55) have found that the conversion of glycerol to glycogen in rat liver involves an asymmetrical handling of the glycerol molecule. The glycerokinase reaction is almost certainly the first step in this reaction sequence, as shown by Bublitz and Kennedy (12).

Conversion of L-\alpha-GP to Phosphatidic Acids

In an attempt to identify the lipid products formed from $L-\alpha$ -GP in the rat liver mitochondrial enzyme system, Kennedy (28) isolated fractions of very high specific activity by chromatography on ion exchange resins, and came to the tentative conclusion that these were phosphatidic acids. The important experiments of Kornberg and Pricer (39, 40) have elucidated the mechanism of formation of phosphatidic acids from $L-\alpha$ -GP.

These workers have distinguished two separate enzymatic processes in phosphatidic acid formation. In the first of these, long-chain fatty acids, such as stearic acid, are activated to form thioesters of CoA. In view of the recent work of Berg (9) it appears probable that this activation proceeds as follows:

[2] RCOOH + ATP
$$\leftarrow$$
 RCO-O-P-O-adenosine + P-O-P, OH

[3] RCO-O-P-O-adenosine + CoA-SH \leftarrow RCO-S-CoA + AMP.

This activation reaction is an essential preliminary not only for phosphatidic acid formation, but probably for all types of esterification reactions and also for fatty acid oxidation (42).

The long-chain fatty acid thioesters of CoA then react with L- α -GP to form a phosphatidic acid (Reaction [4] of Fig. 1). The enzyme system responsible for the esterification of L- α -GP has not been extensively purified, and it is not known whether the primary or the secondary hydroxyl group of L- α -GP is esterified first, or whether both are attacked simultaneously.

Function of Phosphatidic Acids

In 1927, Chibnall and Channon (17) isolated purified preparations of phosphatidic acid from cabbage leaves. The later work of Hanahan and Chaikoff (24) showed that the phosphatidic acid of cabbage leaves arose from the hydrolytic cleavage of choline (and other nitrogenous bases) from

glycerophosphatides. These investigators demonstrated the presence of a phospholipid-splitting enzyme (lecithinase C) in cabbage leaves, which attacks lecithin at the phosphorus-choline linkage, yielding phosphatidic acid and free choline as products. The presence of phosphatidic acids in extracts of cabbage leaves could thus be accounted for on the basis of the action of degradative enzymes.

The work described above makes it clear that in mammalian tissues, phosphatidic acids are not simply products of hydrolysis, but may be produced by separate synthetic pathways. Kornberg and Pricer (40) reported that enzyme preparations from guinea pig liver carry out the esterification of L- α -GP at a vigorous rate. In the experiments of Kennedy (28) using P_i^{32} as a label in experiments with isolated rat liver mitochondria, it was found that the specific activity of the phosphatidic acid fraction was almost two hundred times higher than that of the nitrogen-containing phospholipids, which constituted by far the bulk of the phospholipids present. Similarly, Dawson (18) has found that the specific activity of the phosphatidic acid fraction of brain suspensions after incubation with P_i^{32} was much higher than any other phospholipid fraction.

These results naturally lead to the question of what function phosphatidic acids may carry out in lipid metabolism. Little attention has been paid to these compounds, which do not constitute a quantitatively important fraction of the lipids of animal tissues. One obvious possibility is that phosphatidic acids may represent a stage in the biosynthesis of glycerophosphatides; this hypothesis will be considered more fully in a later section of this paper.

The Function of Cytidine Nucleotides in the Biosynthesis of Phospholipids

In 1952, Kornberg and Pricer (38) reported experiments in which phosphorylcholine, doubly labeled with P³² and C¹⁴, was converted in the presence of a rat liver enzyme into a phospholipid which was not further characterized. The ratio of P³² to C¹⁴ in the lipid product closely approximated that of the labeled phosphorylcholine. On the basis of these results, it was suggested (27, 37) that phosphorylcholine may be incorporated as a unit into the lecithin molecule by the following reaction:

The incorporation of phosphorylcholine into the phospholipids of a guinea pig liver enzyme preparation has also been observed by Rodbell and Hanahan (50) who identified the lipid product as lecithin by isolation of a radioactive product after chromatography on alumina (25). Chromatographic procedures of this type have recently been found (31) to be insufficient to rule out the presence of trace contaminants of high specific activity. Accordingly, the lipid product enzymatically synthesized from phosphorylcholine was treated with alkali as described by Dawson (18) and radioactive L- α -glycerophosphorylcholine isolated (33). These results indicate conclusively that lecithin is the product of this enzymatic reaction.

Cofactor Requirements for Phosphorylcholine Incorporation

In an investigation of the mechanism of the incorporation of phosphorylcholine into the lecithin of isolated rat liver mitochondria, Kennedy and Weiss (32) found that significant incorporation occurred only when the enzyme preparations were supplemented with large amounts of ATP. This requirement for ATP persisted even when synthetic phosphatidic acid was added, suggesting that the actual enzymatic reaction is considerably more complex than the postulated Reaction [4]. The ATP used in these experiments was an amorphous preparation (Pabst, Lot 116), of approximately 95% purity. When crystallized ATP (Pabst, Lot 122) was substituted, no activation was observed (Table I). Closer examination of the cofactor requirement revealed that the true cofactor is CTP. The activity of amorphous preparations of ATP may be attributed to the presence of small amounts (less than one per cent) of CTP. The enzyme system is highly specific in its requirement for the cytidine nucleotide; no other nucleotide of a number tested has been found to be active.

TABLE I

COFACTOR REQUIREMENT FOR PHOSPHORYLCHOLINE INCORPORATION

Added cofactors	Lecithin synthesized (total counts)
(1) 5 μM. of ATP lot 116	590
(2) 5 μM. of ATP lot 122	20
(3) 5 μ M. of ATP lot 122 + 0.5 μ M. ITP	0
(4) 5 μ M. of ATP lot 122 + 0.5 μ M. UTP	50
(5) 5 μ M. of ATP lot 122 + 0.5 μ M. GDP	57
(6) 5 μ M, of ATP lot 122 + 0.5 μ M, CTP	1677
(7) 5 μM. of ATP lot 122 + 0.5 μM. CTP + 2.5 μM. inorganic pyrophosphate	750

Each tube contained 10 μ M.MgCl₂, 50 μ M. of phosphate buffer, pH 7.4, 3 μ M. of phosphorylcholine-P²² (113,000 counts/ μ M.) and 25 mgm. of lyophilized mitochondria in a total volume of 1.0 ml. Incubated at 37° for one hour.

Mechanism of Activation of Phosphorylcholine

The discovery of the requirement of CTP for the conversion of phosphorylcholine to lecithin is the first demonstration of a specific role of a cytidine nucleotide in a major metabolic reaction. The brilliant work of Leloir and his collaborators (13, 15, 41) and of Kalckar and his school (27) has given us much information on the function of uridine-containing coenzymes in carbohydrate metabolism, and of the mechanism of biosynthesis of these extremely important compounds. The possibility therefore arose that phosphorylcholine (and other phosphorylated bases) might react with CTP to form cytidine coenzymes structurally analogous to the uridine series.

The enzymatic synthesis of uridine diphosphate glucose has been described by Munch-Petersen *et al.* (45) by the following reaction:

[5] UTP + α -glucose-1-phosphate \longleftrightarrow uridine diphosphate glucose + P-O-P.

Reaction [5] is similar to the synthesis of DPN discovered by Kornberg (36).

With these reactions as models, Kennedy and Weiss (32) postulated the following abbreviated reaction scheme for the biosynthesis of lecithin:

[7]
$$Cyt-O-P-O-P-O-P+P^{32}-O-choline \longleftrightarrow cyt-O-P-O-P^{32}-O-choline \longleftrightarrow P-O-P$$

The essential feature of this scheme is the formation of the novel compound cytidine diphosphate choline (P¹-cytidine-5′-P²-choline-pyrophosphate) (Fig. 2). The phosphate monoester group of phosphorylcholine is converted to a pyrophosphate in the nucleotide CDP-choline and is thus "activated" for a subsequent reaction in which both phosphorus and choline are transferred as a unit into the lecithin structure. The cytidine nucleotide portion of the molecule which is presumably released during Reaction [8] may then be rephosphorylated to CTP at the expense of ATP by enzymes of the "nudiki" type (10) which are known to have widespread occurrence. In this fashion, the cytidine nucleotide may act in continuous, catalytic fashion, carrying out a group transfer reaction during the course of which the substrate, phosphorylcholine, is actually built into the coenzyme structure itself, as is the case with the uridine coenzymes.

Several lines of experimental evidence now offer proof of this formulation.

CYTIDINE DIPHOSPHATE CHOLINE

Fig. 2

Activity of Synthetic Cytidine Diphosphate Choline and Cytidine Diphosphate Ethanolamine

Largely as a result of the work of H. G. Khorana and his collaborators (20, 34, 35), a new and valuable technique has been developed for the synthesis by purely chemical means of nucleotide pyrophosphate esters. This technique involves the use of N,N'-dicyclohexylcarbodiimide as a condensing agent;

in the presence of this reagent, phosphoric acid esters react to form doubly esterified pyrophosphates, while the carbodiimide is converted to the insoluble urea.

$$\begin{array}{c} O & O \\ \parallel \\ O - P - O H \\ O H \end{array} + R'N = C = NR' \longrightarrow R - O - P - O - P - O - R \\ O H & O H \end{array}$$

This method of synthesis of substituted pyrophosphates utilizes very mild conditions, and is unique in that it can be carried out in the presence of water, aqueous pyridine being a very suitable solvent (34).

When a mixture of two different phosphoric acid esters,

is reacted with the carbodiimide reagent, the expected products should include the two symmetrical pyrophosphates,

and the unsymmetrical

The carbodiimide method is ideally suited for the synthesis of CDP-choline and related compounds (32). The reaction of CMP with an excess of phosphorylcholine leads to the formation of CDP-choline in yields of 40-50% based on CMP. The product may be obtained in a state of analytical purity by means of chromatography on Dowex-1 formate resin.

The availability of synthetic radioactive CDP-choline, prepared from phosphorylcholine-1,2-C¹⁴, made it possible to test the reaction mechanism shown in [7] and [8]. When labeled CDP-choline was incubated with enzyme extracts from liver, it was converted to radioactive lecithin at rates exceeding those observed with CTP + phosphorylcholine, and in yields approaching 1 mole of lecithin per mole of CDP-choline added.

It can be readily seen that reactions of the type shown in [7] and [8] need not be limited to the synthesis of lecithin, but may be applied to the synthesis of any of the glycerophosphatides. For this reason, it was of interest to synthesize radioactive CDP-ethanolamine, by the same methods used for the choline compound. CDP-ethanolamine so prepared has been tested (33) and found to be a precursor of phosphatidyl ethanolamine in a reaction completely analogous to the conversion of CDP-choline to lecithin.

These results strongly suggest that the cytidine nucleotide pathway is a general one, and one may expect that phosphatidyl serine and the acetal phosphatides may be synthesized in an analogous fashion. Further, the sphingomyelin molecule also contains the group

which may also have its origin in CDP-choline. It should be emphasized, however, that no experimental evidence for the formation of phospholipids other than lecithin and phosphatidyl ethanolamine by such a mechanism has yet been obtained.

Isolation of CDP-choline and CDP-ethanolamine from Liver and Yeast

If CDP-choline and CDP-ethanolamine are normally occurring coenzyme forms of these bases, and are implicated in the biosynthesis of phospholipids in vivo, it is to be expected that they should be found in significant amounts in tissues such as liver which carry out the biosynthesis of phospholipids at a relatively high rate. The availability of synthetic radioactive CDP-choline and CDP-ethanolamine makes the analysis of tissues for these compounds a relatively simple matter. Known amounts of radioactive CDP-choline and CDP-ethanolamine are added to deproteinized extracts of freshly removed liver, and then these compounds are reisolated by chromatography on ion exchange resins. The amount of unlabeled CDP-choline and CDP-ethanolamine can then be easily calculated by the isotope dilution observed. When experiments of this kind are carried out on fresh rat liver, it is found that CDP-choline is present to the extent of about 10 micromoles per 100 gm. wet weight. Comparable amounts of CDP-ethanolamine are also found.

CDP-choline and CDP-ethanolamine have also been found in crude preparations of nucleotides from yeast, although accurate determinations of the concentration of these substances in vigorously respiring, growing yeasts have not been made.

It may be concluded that CDP-choline and CDP-ethanolamine occur widely distributed in nature, offering further evidence that these compounds are the precursors of phospholipids in vivo.

Enzymatic Synthesis of CDP-choline and CDP-ethanolamine

Enzymes have been found (33) in liver, in yeast, and in carrot root which carry out the enzymatic synthesis of CDP-choline and CDP-ethanolamine according to Reaction [7]. Using the liver enzyme, conditions can be arranged so that considerable net synthesis of CDP-choline takes place. The enzymatically synthesized CDP-choline has been isolated by chromatography on Dowex-1 formate and its properties found to be identical with the synthetic

material. The enzymatically synthesized CDP-choline is likewise converted to lecithin rapidly and in high yield upon incubation with enzyme extracts from liver.

The demonstration of the enzyme catalyzing Reaction [7] in such widely different sources as liver, yeast, and carrot root indicates that this pathway for the biosynthesis of lecithin must be of almost universal occurrence.

Pyrophosphorolysis of CDP-choline

The enzymatic synthesis of DPN by Reaction [6] and of UDP-glucose by Reaction [5] is readily reversible. It accordingly is to be expected that the synthesis of CDP-choline by Reaction [7] should likewise be reversible. Evidence supporting this conclusion was first afforded by the observation that the addition of small amounts of pyrophosphate markedly reduced the over-all incorporation of phosphorylcholine (Table I). When labeled CDP-choline is incubated with enzyme systems catalyzing Reaction [7] some purely hydrolytic cleavage to CMP + phosphorylcholine occurs. The addition of inorganic pyrophosphate greatly increases the amount of CDP-choline split, indicating the pyrophosphorolysis of CDP-choline and the reversibility of Reaction [7].

UDP-choline, ADP-choline, and GDP-choline

Since only CTP is effective in promoting the over-all incorporation of phosphorylcholine into lecithin, it follows that either Reaction [7] or [8] or both must exhibit a high degree of nucleotide specificity. To test the specificity of Reaction [8] UDP-choline, ADP-choline, and GDP-choline were synthesized in labeled form and found to be completely inactive, using enzyme systems from yeast and from liver which showed high activity toward CDP-choline. Reaction [8] is therefore completely specific for the cytidine compound.

Nature of the Lipid Acceptor Compound

It has not yet been possible to study the transformation of CDP-choline to lecithin in enzyme preparations completely free of endogenous lipid. Dialysis is ineffective in removing lipids and treatment with organic solvents inactivates the enzyme. Largely for this reason, the lipid acceptor compound involved in Reaction [8] has not yet been identified.³ The net reaction shown in [8] consists of an enzymatic transfer of the phosphorylcholine group of CDP-choline to an α,β -diglyceride, but it is by no means certain that the actual mechanism is so simple. Efforts to increase the synthesis of lecithin from CDP-choline by the addition of α,β -diglycerides have been unsuccessful. However, diglycerides with fatty acids of 16–18 carbon atoms have almost no solubility in water, and the added substrate may never penetrate to the site of the enzymatic reaction.

 3NOTE ADDED IN PROOF: The lipid acceptor has now been definitely identified as a D- $\alpha,\,\beta\text{-diglyceride}.$

The rapid enzymatic synthesis of phosphatidic acids which is known to occur in liver extracts makes it necessary to consider whether the lipid acceptor in Reaction [8] is a phosphatidic acid. It is certain that both the phosphorus and choline of lecithin arise from CDP-choline. Therefore, if phosphatidic acids give rise to the rest of the molecule, then the phosphorus of the phosphatidic acid must be eliminated at some stage in the reaction. An attractive possibility would be the dephosphorylation of an L-phosphatidic acid by a phosphatase, furnishing a D- α , β -diglyceride for Reaction [8]. A more complex participation of a phosphatidic acid, involving a direct reaction with CDP-choline and a simultaneous removal of the phosphate group of the phosphatidic acid is by no means ruled out.

It may be noted that Rodbell and Hanahan (50) have reported a slight stimulation of the incorporation of phosphorylcholine into lecithin upon the addition of either phosphatidic acid or α,β -diglyceride to enzyme preparations from guinea pig liver.

Enzymatic Phosphorylation of Choline and Ethanolamine

Phosphorylcholine and phosphorylethanolamine are now known to be precursors of lecithin and phosphatidyl ethanolamine respectively. The question then arises as to the origin of the phosphorylated bases themselves. The work of MacFarlane (43, 44) has shown that an active enzyme in filtrates from cultures of *Clostridium welchii* splits lecithin to form phosphorylcholine and an α,β -diglyceride. The presence of similar enzymes in mammalian tissues might give rise to phosphorylcholine and phosphorylethanolamine, but would not provide a net synthesis of these compounds.

Wittenberg and Kornberg (56) have described an enzyme present both in yeast and in liver which catalyzes the transfer of phosphate from ATP to choline:

The purified yeast enzyme also phosphorylates ethanolamine, but only at relatively high concentrations of this substrate. The work of Ansell and Dawson (1) suggests that ethanolamine is phosphorylated in minces of brain tissue.

It seems reasonable to conclude that the net synthesis of phosphorylcholine and phosphorylethanolamine takes place according to Reaction [10].

Function of L-\alpha-Glycerophosphorylcholine

In 1945, Schmidt, Hershman, and Thannhauser (53) isolated L-\alpha-glycerophosphorylcholine from autolyzed pancreas. Subsequent investigations (14, 52) have shown that this compound and L-\alpha-glycerophosphorylethanolamine are present apparently as normal constituents in a number of tissues. It has been suggested that these diesters are precursors of glycerophosphatides which could be formed from them by esterification in an enzymatic reaction which would presumably utilize long-chain fatty acid thioesters of CoA. No evidence in support of this hypothesis has been obtained in studies with cell-free enzyme systems.

A recent investigation by Dawson (19) in which the specific activities of L- α -glycerophosphorylcholine and L- α -glycerophosphorylethanolamine were measured at various times after the injection of labeled phosphate *in vivo* also leads to the conclusion that these compounds are not precursors of phospholipids.

The Incorporation of Free Choline into the Lipids of Mitochondria

A rapid incorporation of free choline into the lipids of isolated mitochondria was noted by Kennedy (29). Neither phosphorylcholine nor L- α -glycerophosphorylcholine are intermediates in this reaction, for which CoA and ATP are necessary cofactors (30).

The product derived from choline was identified as lecithin since it was found to be (a) a lipid, (b) labile to alkali in the procedure of Hack, and (c) indistinguishable from lecithin when chromatographed on alumina by a method based on that of Hanahan (25). However, more recent work (31) has shown that this identification was erroneous.

The finding that ATP and CoA were cofactors for the reaction made it necessary to examine more closely the possibility that the product might in fact be a long-chain fatty acid ester of choline. Accordingly, palmitoylcholine-1,2-Cl4 was synthesized by chemical methods and its properties compared with that of the product of the enzymatic reaction. Surprisingly, it was found that palmitoylcholine could not easily be separated from lecithin by chromatography on alumina, and its other properties were quite similar to the labeled product.

Chromatography of the enzymatically synthesized radioactive lipid on cation-exchange resins led to the isolation of highly active lipid material in a fraction completely free of phosphorus, from which it can be definitely stated that it is not lecithin. Not enough material has as yet been obtained for complete analysis, but it seems likely that it is a long-chain fatty acid ester of choline.

Ethanolamine is also rapidly converted to a lipid in this system. Longchain fatty acid esters of ethanolamine and choline apparently have not been described as naturally-occurring lipids, and it is not known what physiological function may be assigned to this enzyme system, if in fact it is catalyzing the synthesis of these esters.

Discussion

The discovery of the role of the cytidine coenzymes in the biosynthesis of phospholipids opens further avenues of research which can only briefly be mentioned here. In the first place, it would be of interest to determine whether phospholipids other than lecithin and phosphatidyl ethanolamine are synthesized by this enzymatic pathway. The biosynthesis of sphingomyelin, of phosphatidyl serine, and of certain of the inositol phosphatides as well as of the acetal phosphatides may be formulated as taking place by an essentially similar mechanism, but evidence on these points is lacking.

Further, it may prove of value to investigate whether compounds such as CDP-choline take part in metabolic reactions other than those leading to the synthesis of phospholipids. In this connection, the important but little understood lipotropic activity of choline comes to mind. Is it necessary for choline to be converted to CDP-choline in order to exert its lipotropic effect? It will remain for future research to decide such questions. It may be noted that in the case of uridine diphosphate glucose a number of metabolic reactions, such as oxidation to glucuronic acid, which one might expect the free sugar to undergo, actually take place only when the sugar is incorporated into the nucleotide. If an analogous situation holds with the cytidine compounds, one might anticipate that CDP-choline would be the active form of choline in a variety of biological reactions.

Finally, a new and unexpected link has appeared between phospholipid metabolism and the function of ribonucleic acid. For many years, the adenosine coenzymes virtually dominated the biochemical arena. ATP was found to provide the driving force for so many reactions that there was some danger that it would come to be regarded as a kind of philosopher's stone capable of effecting every kind of biosynthesis. We now know that specific coenzymatic functions must be ascribed to all of the 5'-ribotides found in ribonucleic acid. This important fact must be taken into account in any attempt to explain the vitally important functions of ribonucleic acid in every kind of living cell.

References

1. Ansell, G. B. and Dawson, R. M. C. Biochem. J. 50: 241. 1951.

Автом, С. In Phosphorus metabolism. Vol. II. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1952.

3. ARTOM, C. Ann. Rev. Biochem. 22:211. 1953.

- BAER, E. and KATES, M. J. Biol. Chem. 175: 79. 1948.
 BAER, E. and KATES, M. J. Biol. Chem. 185: 615. 1950.
 BAER, E. and KATES, M. J. Am. Chem. Soc. 72: 942. 1950.

- 7. BAER, E., MAURUKAS, J., and RUSSELL, M. J. Am. Chem. Soc. 74: 152. 1952.
- 8. BARKER, H. A. and LIPMANN, F. J. Biol. Chem. 179: 247. 1949.

9. BERG, P. J. Am. Chem. Soc. 77: 3163. 1955.

- 10. BERG, P. and JOKLIK, W. K. J. Biol. Chem. 210: 657. 1954.
- 11. Bublitz, C. and Kennedy, E. P. J. Biol. Chem. 211: 851. 1954.
- 12. Bublitz, C. and Kennedy, E. P. J. Biol. Chem. 211:963. 1954.
- CABIB, E., LELOIR, L. F., and CARDINI, C. E. J. Biol. Chem. 203: 1055. 1953.

14. CAMPBELL, P. N. and WORK, T. S. Biochem. J. 50: 449. 1951-52.

- 15. CAPUTTO, R., LELOIR, L. F., CARDINI, C. E., and PALADINI, A. C. J. Biol. Chem. 184: 333. 1950.
- 16. CHAIKOFF, I. L. and ZILVERSMIT, D. B. Advances in Biol. and Med. Phys. 1:321. 1948.
- 17. CHIBNALL, A. C. and CHANNON, H. J. Biochem. J. 21: 233. 1927.
- 18. Dawson, R. M. C. Biochim et Biophys. Acta, 14: 374. 1954.

19. Dawson, R. M. C. Biochem. J. 59: 5. 1955.

- 20. DEKKER, C. A. and KHORANA, H. G. J. Am. Chem. Soc. 76: 3522. 1954.
- FISHLER, M. C., TAUROG, A., PERLMAN, I., and CHAIKOFF, I. L. J. Biol. Chem. 141: 809. 1941.
- 22. FRIEDKIN, M. and LEHNINGER, A. L. J. Biol. Chem. 177: 775. 1949.
- 23. FRIES, B. A., SCHACHNER, H., and CHAIKOFF, I. L. J. Biol. Chem. 144: 59. 1942.
- 24. HANAHAN, D. J. and CHAIKOFF, I. L. J. Biol. Chem. 172: 191. 1948.

25. HANAHAN, D. J., TURNER, M. B., and JAYKO, M. E. J. Biol. Chem. 192: 623. 1951.

26. KALCKAR, H. M. Enzymologia, 2: 47. 1937.

27. KALCKAR, H. M. and KLENOW, H. Ann. Rev. Biochem. 23: 527. 1954.

 Kennedy, E. P. J. Biol. Chem. 201: 399. 1953.
 Kennedy, E. P. J. Biol. Chem. 209: 525. 1954. 30. Kennedy, E. P. Federation Proc. 13: 241. 1954.

31. KENNEDY, E. P. Unpublished.

32. KENNEDY, E. P. and WEISS, S. B. J. Am. Chem. Soc. 77: 250. 1955. 33. KENNEDY, E. P. and WEISS, S. B. J. Biol Chem. In press.

34. KHORANA, H. G. J. Am. Chem. Soc. 76: 3517. 1954.

35. KHORANA, H. G. and TODD, A. R. J. Chem. Soc. 2257. 1953. 36. KORNBERG, A. J. Biol. Chem. 182: 779. 1950.

- KORNBERG, A. In Phosphorus metabolism. Vol. II. Edite. B. Glass. The Johns Hopkins Press, Baltimore, Md. 1952 Edited by W. D. McElroy and
- 38. Kornberg, A. and Pricer, W. E., Jr. Federation Proc. 11: 242. 1952.
- 39. KORNBERG, A. and PRICER, W. E., Jr. J. Biol. Chem. 204: 329. 1953. 40. KORNBERG, A. and PRICER, W. E., Jr. J. Biol. Chem. 204: 345. 1953.
- Leloir, L. F. and Cardini, C. E. J. Am. Chem. Soc. 75: 6084. 1953.
 Lynen, F. Ann. Rev. Biochem. 24: 653. 1955.

43. MACFARLANE, M. G. Biochem. J. 42: 587. 1948.

44. MacFarlane, M. G. and Knight, B. C. J. G. Biochen. J. 35: 884. 1941. 45. Munch-Petersen, A., Kalckar, H. M., Cutolo, E., and Smith, E. E. B. Nature, 172:1036. 1953.

46. OGSTON, A. G. Nature, 162: 963. 1948. 47. Рорјак, G. and Muir, H. Biochem. J. 46: 103. 1950.

48. POTTER, V. R. and HEIDELBERGER, C. Nature, 164: 180. 1948.

49. RILEY, R. F. J. Biol. Chem. 153: 535. 1944.

50. RODBELL, M. and HANAHAN, D. J. Biol. Chem. 214: 607. 1955.

- 51. Schambye, P., Wood, H. G., and Popják, G. J. Biol. Chem. 206: 875. 1954.
 52. Schmidt, G., Greenbaum, L. M., Fallot, P., Walker, A. C., and Thannhauser, S. J. J. Biol. Chem. 212: 887. 1955.
- Schmidt, G., Hershman, B., and Thannhauser, S. J. J. Biol. Chem. 161: 523. 1945.
 Schwartz, P. and Carter, H. E. Proc. Natl. Acad. Sci. U.S. 40: 499. 1954.

55. Swick, R. W. and Nakao, A. J. Biol. Chem. 206: 883. 1954.

56. WITTENBERG, J. and KORNBERG, A. J. Biol. Chem. 202: 431. 1953.

57. ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. J. Biol. Chem. 176: 193. 1948. 58. ZILVERSMIT, D. B., ENTENMAN, C., and FISHLER, M. C. J. Gen. Physiol. 26: 325. 1943.

DISCUSSION: G. C. BUTLER^{1, 2}

Dr. Kennedy has given us a comprehensive survey of the mechanisms of all the coupling reactions necessary for the biological synthesis of phospholipids. I should like to bring up for discussion the complex question of the order in which these reactions take place by asking Dr. Kennedy to comment on the probable identity of the lipid acceptor of phosphorylcholine. A number of reactions are theoretically possible:

[1] CDP-choline + triglyceride → CMP + fatty acid + lecithin, or CMP - fatty acid + lecithin,

[2] CDP-choline $+ \alpha$, β -diglyceride \rightarrow CMP + lecithin,

- [3] CDP-choline + phosphatidic acid → CMP + P_i + lecithin, or CDP + lecithin,
- [4] CDP-choline + phosphatidyl ethanolamine → CDP-ethanolamine + lecithin.

One reason for being interested in possibilities [1], [2], and [3] is that these might provide a link between triglyceride and phospholipid metabolism. Is it possible that phospholipid is an obligatory intermediate in either the anabolism or catabolism of triglyceride? It is perhaps relevant that Kornberg and Pricer's fatty acid esterifying system could not react with free glycerol.

¹Contribution from the Department of Biochemistry, University of Toronto, Toronto, Ontario. ³CMP = cytidine-5'-monophosphate; CDP = cytidine-5'-diphosphate.

GENERAL DISCUSSION

Dr. E. P. Kennedy.—All attempts to identify an α,β -diglyceride or a phosphatidic acid with the acceptor of the phosphorylcholine group have so far met with failure.

Dr. Marinetti.—Although Dr. Kennedy's work indicates that phosphatidic acids are synthesized in cell free systems, and Dr. Berry's work demonstrates that these lipids are formed in brain slices and homogenates, studies in our laboratory on tissue lipids obtained from rats injected with P₂₂ failed to show the presence of even trace amounts of phosphatidic acids. As pointed out by Dr. Witter yesterday, the chromatographic systems used in our studies were the same as those employed by Dr. Berry. Since lecithin, cephalin, serine phosphatide, acetal phosphatide, possibly inositol phosphatide, and other unidentified phospholipids were labelled and since we used large amounts of the total lipid extract for the chromatographic analysis, any labelled phosphatidic acid should have been detected. Dr. Kennedy has mentioned that a pure sample of phosphatidic acid added to in vitro systems is apparently inactive for the synthesis of lecithin via cytidine diphosphate choline. It is possible that one of the unidentified phospholipids observed on our chromatograms may be the lipid acceptor which reacts with cytidine diphosphate choline to form lecithin. Although phosphatidic acids are synthesized in vitro, the physiological significance of this synthetic pathway must be regarded cautiously.

⁴ See footnote page 343.

METABOLISM OF PHOSPHOLIPIDS IN VITRO

By L. E. HOKIN AND MABEL R. HOKIN

The subject which we are to discuss today might more appropriately be entitled, "Certain aspects of the metabolism of phospholipids in vitro", because it concerns the relationship between one particular type of phospholipid metabolism and one specific physiological function, i.e. protein secretion. There is really no pre-existent literature on this subject, as it arose accidentally out of some studies we were carrying out on the incorporation of P32 into ribonucleic acid (RNA) in pancreas slices. It may be of interest to relate how we were introduced into the field of phospholipid metabolism.

We were primarily interested in the relationship between RNA metabolism and protein synthesis, as studied in pancreas slices. Early in our work we saw a paper by Guberniev and Il'ina (12) in which it was shown that when secretion was stimulated in digestive glands (pancreas and salivary glands) of anesthetized dogs, there were marked increases in the incorporation of P32 into the "nucleoproteins". These "nucleoproteins" were reported to have been isolated by the method of Schmidt and Thannhauser (31), which is really designed to isolate RNA and desoxyribonucleic acid (DNA) fractions and not nucleoprotein, but Guberniev and Il'ina gave no details of their fractionation procedures.

Guberniev and Il'ina concluded from their studies that nucleic acid renewal and protein synthesis are interrelated. This conclusion was based on the assumption that when the secretion of digestive enzymes was stimulated the formation of new enzyme was accelerated. One of us (13) had already demonstrated that slices of pigeon pancreas, when incubated in bicarbonate saline, were capable of synthesizing amylase. If acetylcholine was added, part of the preformed enzyme was actively secreted into the medium. This was shown to be true secretion by its dependence on the energy of respiration and its complete inhibition by low concentrations of the anticholinergic agent atropine (23). There was some discharge of enzyme in the absence of acetylcholine, but this cannot be regarded as secretion because it was not abolished by anaerobiosis. We use the term "secretion" here to refer exclusively to the process of active extrusion*, although it is frequently used to include the formation as well as the active extrusion of a secretory product.

In pancreas slices we had observed that the formation and secretion of the digestive enzymes were independent processes (13, 32). Either process could be stimulated by specific means without affecting the other.

¹Manuscript received December 1, 1955.

Contribution from the Department of Pharmacology, McGill University, Montreal, Quebec. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955.

^aM. A. Abercrombie, C. J. Hickman, and M. L. Johnson in A dictionary of biology, Hunt, Barnard and Co., Ltd., Harmondsworth, Middlesex, England, define secretion as "The passage of (usually complex) material elaborated by a cell from the inside to the outside of its plasma membrane".

We therefore investigated the possibility that the increased incorporation of P32 into the "nucleoproteins", as observed by Guberniev and Il'ina, might be related to the secretory process rather than to the formation of the enzymes. We felt that such a relationship might have far-reaching implications for the function of nucleic acids. To determine the specific activities of the RNA the tissue was fractionated according to the method of Schmidt and Thannhauser (31), and the resulting ribonucleotides were further purified by paper chromatography (28). It was found that the stimulation of enzyme secretion by acetylcholine or by carbamylcholine (this agent has identical actions to those of acetylcholine but is not hydrolyzed by cholinesterase) was associated with a 50 to 100% increase in the rate of incorporation of P32 into the "RNA" (14). At about this time studies began to emerge from various laboratories (4, 5, 11, 33, 27) which indicated that in many tissues the "RNA" fraction of Schmidt and Thannhauser was grossly contaminated with inositol-containing phospholipids as well as other phosphorus-containing substances. The possibility therefore arose that the stimulation of P32 incorporation into "RNA" was really into some phosphorus-containing compound other than RNA. Rigorous purification of RNA by the technique of Davidson and Smellie (4), which had in the meantime become available, showed that this must be the case (16, 17), since the incorporation of P³² into RNA was not then found to be affected by acetylcholine. The incorporation of P32 into the ether-soluble phospholipids was, however, greatly stimulated by acetylcholine (23, 24). Subsequent work showed that this stimulation was due to an increased turnover of phosphate in preformed phospholipids and not to synthesis of phospholipid de novo, as will be shown later. But first we would like to review the evidence connecting phospholipids with the secretion (active extrusion) of complex molecules from the cell and other examples of the phospholipid effect which have been observed.

The Role of Phospholipids in Protein Secretion

 Enzyme Secretion and the Turnover of Phosphate Ester Moieties in the Phospholipids of Pancreas

Stimulation of the vagus nerve or injection of cholinergic drugs into animals gives rise to the secretion of a viscous material by the pancreas which is rich in enzymes. Histological examination of the pancreas shows marked depletion of the zymogen granule content. Cholinergic stimulation gives rise to little or no secretion of water and bicarbonate (see Babkin (2)). The secretion of these latter substances is under control of the hormone, secretin. The incubation of pancreas slices with cholinergic drugs is thus a fairly specific means of stimulating enzyme secretion without giving rise to any appreciable secretion of other substances. When enzyme secretion was stimulated in pancreas slices by the addition of either acetylcholine or carbamylcholine there was a 5- to 10-fold increase in the incorporation of P32 into the total ether-soluble phospholipids (23). Respiration and the incorporation of P32 into the acid-soluble phosphate ester fraction were unaffected by cholinergic agents, so it can be concluded that the phospholipid effect was not secondary

to increased metabolism of the tissue or to an increased permeability of the cells to phosphate ions. Low concentrations of atropine completely abolished the stimulatory effects of acetylcholine on both enzyme secretion and the incorporation of P³² into the phospholipids. There was no change in the concentration of ether-soluble phospholipid in the tissue (24), and it has recently been found that no measurable quantity of phospholipid is secreted by the pancreas *in vivo* in response to cholinergic stimulation (15). The incorporation of P³² into the phospholipids and its stimulation by cholinergic agents were dependent on the presence of oxygen. Others (1, 34) have also found that the incorporation of P³² into phospholipids of a variety of tissues is dependent on the energy of respiration. When enzyme synthesis was stimulated by the addition of a mixture of amino acids there was no change in the rate of incorporation of P³² into the phospholipids.

The simplest hypothesis to explain our results is that the phospholipid effect is somehow related to the process of protein secretion. At an early stage of our work we were led away from this hypothesis by two sets of observations (24). One was that the rates of incorporation of P32 into the phospholipids in vivo were exactly the same in pancreases of fed and fasted mice. We were under the erroneous impression that the secretion of enzymes would be greater in fed mice. Subsequent experiments have shown that the secretion of amylase is much the same in pancreases of fed and fasted mice (22). The second line of evidence which we felt argued against the view that the phospholipids played a role in protein secretion was that enzyme secretion and the incorporation of P32 into the phospholipids did not always show a close relationship. For instance, enzyme secretion became maximal when the concentration of acetylcholine reached 10⁻⁶M, but the incorporation of P32 into the phospholipids did not become maximal until the concentration of acetylcholine was raised to 10-5M. Again, maximal enzyme secretion could be obtained by using choline plus eserine as stimulants; under these conditions the incorporation of P32 into the phospholipids was only one-third (It should be pointed out that the cells were not completely depleted of enzyme when this maximal secretion was reached.) The failure to find a parallelism between enzyme secretion and the incorporation of P32 into the phospholipids under all conditions is really no argument against the hypothesis that phospholipids are concerned in enzyme secretion. The important point is that we have yet to find a case where protein secretion is stimulated without an associated stimulation of P32 incorporation into the phospholipids.

The experiments that were most convincing of a functional link between the phospholipid effect and protein secretion were those carried out with pancreozymin. This hormone specifically stimulates enzyme secretion in the pancreas and, as far as is known, has no other function. Pancreozymin gave almost exactly the same picture as acetylcholine. Enzyme secretion was stimulated in pancreas slices and the incorporation of P³² into the phospholipids was markedly increased (20). Pancreozymin did not stimulate respiration or

the incorporation of P³² into the acid-soluble phosphate esters. As was the case with acetylcholine, the concentration of pancreozymin which gave maximal enzyme secretion was lower than that necessary to produce maximal stimulation of the incorporation of P³² into the phospholipids. This indicates that the phospholipid effect is not a consequence of the passage of enzyme out of the cell. It probably means that some step in the secretory process which is secondary to the phospholipid effect is rate-limiting.

The stimulatory effect of submaximal concentrations of acetylcholine and pancreozymin were additive up to the maximum per cent stimulation found with higher concentrations of either agent. No additive effects were obtained if either agent was used in maximal concentration—a reasonable indication

that both agents act on the same type of cell.

Concentrations of atropine which completely abolished the stimulatory effects of acetylcholine on enzyme secretion and the incorporation of P³² into the phospholipids were without effect on the stimulatory effects of pancreozymin. This suggests that pancreozymin combines with a different receptor than that for acetylcholine.

Secretin, which stimulates water and bicarbonate secretion in the pancreas, was without effect on enzyme secretion or the incorporation of P³² into the phospholipids.

 Protein Secretion and the Incorporation of P³² into the Phospholipids of Salivary Glands

There are other digestive glands where the relationship between protein secretion and the incorporation of P³² into the phospholipids may be studied. Among these the salivary glands were found to be particularly suitable (21). The submaxillary gland secretes mucoprotein in response to either cholinergic or adrenergic stimulation. We were able to develop a method for quantitatively measuring mucin secretion from slices of submaxillary gland incubated in vitro. This method was based on the fact that submaxillary mucin, which could be precipitated with deproteinizing agents, contains chondrosamine (galactosamine) (3). This was liberated by hydrolysis and was measured colorimetrically by the method of Elson and Morgan (9).

Addition of acetylcholine or adrenalin to the media in which the slices were incubated approximately doubled the quantity of mucin extruded into the medium. Acetylcholine increased the incorporation of P³² into the phospholipids sixfold, and adrenalin increased this incorporation about two-to three-fold.

Amylase secretion and the incorporation of P³² into the phospholipids were studied in slices of guinea pig parotid and rabbit parotid. Acetylcholine stimulated amylase secretion in slices of parotid gland, as would be expected from previous physiological studies. The incorporation of P³² into the phospholipids of secreting slices was approximately four times greater than into the phospholipids of non-secreting slices. To our surprise, adrenalin also stimulated enzyme secretion in parotid slices and increased the incorporation of P³² four- to five-fold.

In summary, cholinergic agents and pancreozymin in the pancreas and acetylcholine and adrenalin in the salivary glands each stimulate the secretion of protein and in each case this is accompanied by an increased incorporation of P³² into the phospholipids.

3. Possible Phospholipid Effects in Other Glandular Tissues

It would be of value to determine whether the stimulation of secretion in other glandular tissues would be associated with an increased incorporation of P³² into the phospholipids. In this connection a paper published by Morton and Schwartz (29) in 1953 is of interest. These workers found that thyrotropic hormone stimulated the incorporation of P³² into the phospholipids of beef thyroid slices without affecting the incorporation of P³² into the acid-soluble phosphate ester fraction. Thyrotropic hormone stimulates hyperplasia of the thyroid in vivo and this could bring about increased phospholipid synthesis (see Johnson and Albert (25)). But it is unlikely that during the short incubation periods any significant hyperplasia of the thyroid would take place. It is much more likely that the increased incorporation of P³² into the phospholipids in thyroid slices incubated in the presence of thyrotropic hormone is associated with a stimulation of the discharge of colloid. This may therefore be another example of a functional relationship between phosphate turnover in the phospholipids and protein secretion.

Effects of Acetylcholine on the Turnover of Phosphate in the Phospholipids of Guinea Pig Brain Cortex Slices

The effects of acetylcholine or carbamylcholine on the incorporation of P32 into the phospholipids were tested in a variety of tissues (23). No stimulation was observed in slices of pigeon liver, guinea pig liver, kidney cortex and heart ventricle, or pigeon gizzard (smooth muscle). stimulation was obtained in slices of pigeon brain and guinea pig brain cortex (23, 18). This stimulation increased linearly with the logarithm of the acetylcholine concentration up to 10-2M, the minimum effective concentration of acetylcholine being between $10^{-6}M$ and $10^{-6}M$. The stimulation of the incorporation of P32 into the phospholipids of brain cortex slices was abolished by very low concentrations of atropine (ca. 10-6M), but high concentrations of atropine stimulated the incorporation of P32 into the phospholipids. This effect of high concentrations of atropine was not observed in pancreas slices. It is generally assumed that because of its structural similarity to acetylcholine, atropine blocks the action of acetylcholine by combining with the acetylcholine receptor. However, in the case of the phospholipid effect in brain cortex, atropine, presumably because of this structural similarity, appears to be able at higher concentrations to mimic the action of acetylcholine.

What is the significance of the stimulation of phosphate turnover in the phospholipids of brain cortex? Inasmuch as the evidence from other tissues indicates that this phospholipid effect is concerned with protein secretion,

SUMMARY OF THE EVIDENCE RELATING TO THE PHOSPHOLIPID EFFECT IN VARIOUS ORGANS

Tissue	Stimulant	Material secreted*	Average % increase in P** incorporation into phospholipids†
Pigeon pancreas Pigeon pancreas	Pancreozymin Acetylcholine Carbamylcholine Pilocarpine	Digestive enzymes Digestive enzymes	1000
Rabbit submaxillary gland	Acetylcholine	Mucin	200
Rabbit submaxillary gland	Adrenalin	Mucin	300
Rabbit parotid gland	Acetylcholine	Amylase	400
Rabbit parotid gland	Adrenalin	Amylase	, 400
Beef thyroid‡	Thyrotropic hormone	Colloid?	250
Guinea pig brain cortex	Acetylcholine	۸.	250

*Except in the case of thyroid and brain cortex, the effects of the stimulatory agent on both secretion of the material indicated and the incorporation of Parinto the ether-soluble phospholipids were assayed.

†The specific activity observed in the presence of the stimulatory agent in vitro, expressed as a percentage of the control value.

‡Data taken from Morton and Schwartz (29).

there is no reason at this stage to look for other hypotheses to account for the phenomenon in brain cortex. The nature of the effect in brain is very similar to that in pancreas, as will be shown later. The simplest hypothesis seems to us to be that acetylcholine stimulates the secretion of some complex molecule from certain cells of the brain cortex. The concept of secretion of proteins or polypeptides by neural tissue is not new. It is well established in the neurohypophysis, and in the invertebrate kingdom there are numerous examples of secretion by neural tissue, including the brain.

A summary of the evidence relating to the phospholipid effect in various organs is shown in Table I.

Studies with Cell-free Preparations

If the phospholipid effect could be obtained in cell-free preparations it would be possible to study the mechanism whereby the various stimulatory agents bring about increased turnover of phosphate in the phospholipids. Preliminary experiments with homogenates of guinea pig parotid and pigeon pancreas were unsuccessful. However, it was possible to obtain a stimulation in homogenates of guinea pig brain cortex in response to acetylcholine or carbamylcholine. The incubation conditions were patterned after those of Dawson (6), who has described conditions under which maximal incorporation of P32 into the phospholipids of brain dispersions takes place. The stimulation of P32 incorporation into phospholipids in brain homogenates was 25 to 30% as compared with 100 to 150% in slices. The maximal stimulation occurred with 10-5M acetylcholine; this concentration is less than one thousandth of that required for maximal stimulation in slices, suggesting that the permeability of brain slices to acetylcholine may be very low.

The Nature of the Phospholipid Effect

The fact that the phospholipid level in the stimulated tissue does not increase and that no phospholipid is secreted argued against the possibility that the phospholipid effect was due to de novo synthesis. But to answer this question unequivocally, pancreas slices were incubated with glycerol-1-C14 without and with either acetylcholine (with eserine) or pancreozymin, and similar experiments were carried out with brain cortex slices (24, 18). After incubation the total phospholipid fraction was isolated and hydrolyzed with 5 N KOH for one hour at 75°. After neutralization with perchloric acid the glycerophosphate of the phospholipids was isolated by ionophoresis on paper and its specific activity was determined. The results are shown in Table II. In pancreas slices, although the incorporation of P³² into the glycerophosphatides was increased severalfold by acetylcholine or pancreozymin, there was only a 20 to 30% increase in the incorporation of glycerol-1-C14. In slices of brain cortex the incorporation of glycerol-1-C14 was slightly inhibited although the usual stimulatory effect on P32 incorporation occurred. These results show that the phospholipid effect in pancreas and

TABLE II

Comparison of the incorporation of glycerol-1- C^{14} and P^{32} into the glycerophosphate of glycerophosphatides in the presence of pancreozymin and acetylcholine

		Specific activities (c.p.m./µgm. P)					
Times	Calmulana.	Glycer	rol-1-C14	Pas			
Tissue	Stimulant -	Control	+Stimulant	Control	+Stimulant		
Pancreas	Pancreozymin	21	28	56	292		
Pancreas	Acetylcholine	20	24	114	979		
Brain cortex	Acetylcholine	27	16	257	375		

in brain cortex represents an increased turnover of phosphate in preformed phospholipid and not an increased total synthesis of phospholipid.

In which phospholipid types does this increased turnover of phosphate take place? An answer to this question (19) became possible when Dawson (8) published a chromatographic technique for separating the water-soluble products resulting from mild alkaline hydrolysis of the phospholipids. These hydrolysis products were identified by Dawson as glycerophosphorylcholine, glycerophosphorylethanolamine, glycerophosphorylserine, glycerophosphate, and a substance containing inositol and phosphate. These substances were derived from phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidic acid, and, in the case of brain, diphosphoinositide. In the case of the pancreas we shall refer to the parent substance of the inositol-containing hydrolysis product with the noncommittal term of phosphoinositide, since there is some evidence that the parent compound in the pancreas may not be disphosphoinositide (19).

Table III shows the incorporation of P³² into the above phospholipid types in pancreas slices and brain cortex slices in the presence and absence of acetylcholine. It can be seen that the rates of incorporation of P³² into the

TABLE III

Effects of acetylcholine on the incorporation of P82 and ethanolamine-2-C14 into individual phospholipids

	Specific activities (c.p.m./µgm. P)						
	Brain Pas		Pancreas				
Phospholipid type			Pn		Ethanolamine-2-Ci		
	Control	ACh*	Control	ACh*	Control	ACh*	
Phosphatidyl choline	76	126	41	55	1	2	
Phosphatidyl ethanolamine	9	12	236	433	80	151	
Phosphatidyl serine	2	3	9	25			
Phosphoinositide	710	1650	440	7480			
"Phosphatidic Acid"	1020	2180	754	1250			
Calculated over-all specific activity	70	135	135	782			

^{*} A Ch = acetylcholine.

various phospholipid types are very dissimilar. In the pancreas the highest specific activities were those of "phosphatidic acid", phosphoinositide, and phosphatidyl ethanolamine. The incorporation of P32 into phosphatidyl choline was by comparison quite low, and the incorporation into phosphatidyl serine was extremely low. Acetylcholine stimulated the incorporation of P32 into each of the five phospholipids but to an unequal degree. By far the greatest stimulation occurred in phosphoinositide; the stimulation in this compound accounted for 75% of the over-all stimulation. The specific activities of the "phosphatidic acid" from the pancreas are of doubtful significance, since the glycerophosphate derived from this compound was present in trace amounts. Traces of glycerophosphate derived from any of the other glycerophosphatides as a result of a too extensive hydrolysis would markedly alter the specific activity of "phosphatidic acid". The finding that the major portion of the total stimulation occurred in the inositolcontaining phospholipids readily accounts for our earlier finding of a stimulation in the Schmidt-Thannhauser RNA fraction.

In brain cortex slices the pattern of incorporation of P³² into the various phospholipid types was somewhat different from the pattern in pancreas slices. In brain cortex there was virtually no incorporation of P³² into phosphatidyl ethanolamine or phosphatidyl serine. This agrees with the earlier findings of Dawson (8) in brain "dispersions". However, we found an appreciable incorporation of P³² into phosphatidyl choline of brain cortex slices. Dawson did not find any significant incorporation of P³² into phosphatidyl choline of brain slices (7) or "dispersions" (8), but an appreciable incorporation was observed in brain cortex *in vivo* (7).

Acetylcholine stimulated the incorporation of P³² into phosphatidyl choline by about 50% and into diphosphoinositide and "phosphatidic acid" by approximately 100%.

The basic structure of the glycerophosphatides contains three types of bonds-fatty acid - glycerol ester linkages, a bond between phosphate and glycerol, and a bond between phosphate and choline, ethanolamine, or serine. Recent work (10, 30) has indicated that the basic structure of the monophosphoinositide from soybean is similar to the above; monophosphoinositides could be classified as glycerophosphatides in which inositol occupies a position analogous to the base or amino acid of the other glycerophosphatides. It is obvious from examination of the basic structure of the phospholipids that increased incorporation of P22 without increased incorporation of glycerol-1-C14 must indicate that the essential reaction involves cleavage and resynthesis of the glycerol-phosphate bond but not that of the fatty-acid - glycerol bonds. would therefore be expected that the incorporation of inositol, base, or amino acid into the respective phospholipids would be increased to the same extent as that of the phosphate. This was shown to be the case using ethanolamine-2-C14. In the secreting pancreas the incorporation of ethanolamine and choline (derived from the labelled ethanolamine) into phosphatidyl ethanolamine and phosphatidyl choline, respectively, were increased to approximately the same

extent as the incorporation of phosphate (Table III). Unfortunately we have not as yet been able to obtain any radioactive inositol but by analogy it is likely that the incorporation of inositol is also increased to the same extent as the phosphate. In conjunction with the observation of Kennedy and Weiss (26) that phosphorylcholine is incorporated as a unit into phosphatidyl choline, our studies make it probable that the various phosphate ester moieties involved in the phospholipid effect turn over as units in their respective phospholipids.

References

- Artom, C. In A symposium on phosphorus metabolism. Vol. II. Edited by W. D. McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1952. p. 203.
 Babkin, B. P. Secretory mechanism of the digestive glands. 2nd ed. Paul B. Hoeber, Inc., New York. 1950.
 Blux, G. Sushinghuch, L. and Werdyng, L. Acta Cham. Scand 6 : 259. 1053.
- 3. BLIX, G., SVENNERHOLM, L., and WERNER, I. Acta Chem. Scand. 6: 358. 1952.
- DLIX, G., SVENNERHOLM, L., and WERNER, I. Acta Chem. Scand. 6:
 DAVIDSON, J. N. and SMELLIE, R. M. S. Biochem. J. 52: 594. 1952.
 DAVIDSON, J. N. and SMELLIE, R. M. S. Biochem. J. 52: 599. 1952.
 DAWSON, R. M. C. Biochem. J. 55: 507. 1953.
 DAWSON, R. M. C. Biochem. J. 57: 237. 1954.
 DAWSON, R. M. C. Biochim. et Biophys. Acta, 14: 374. 1954.
 ELSON, J. A. and Moderaty, W. T. D. Biochim. 100. 100. 100. 100. 100.

- DAVIDSON, J. N. and SMELLIE, R. M. S. Biochem. J. 52:599. 1952.
 DAWSON, R. M. C. Biochem. J. 55:507. 1953.
 DAWSON, R. M. C. Biochem. J. 57:237. 1954.
 DAWSON, R. M. C. Biochem. J. 57:237. 1954.
 DAWSON, R. M. C. Biochim. et Biophys. Acta, 14:374. 1954.
 ELSON, L. A. and MORGAN, W. T. J. Biochem. J. 27:1824. 1933.
 FAURE, M. and MORIGE-COULON, M. J. Compt. rend. 236:1104. 1953.
 FOLCH, J. In A symposium on phosphorus metabolism. Vol. II. Edited by McElroy and B. Glass. The Johns Hopkins Press, Baltimore, Md. 1952. p.
 GUBERNIEV, M. A. and IL'INA, L. I. Doklady Akad. Nauk S.S.S.R. 71:351. 19.
 HOKIN, L. E. Biochim. et Biophys. Acta, 8:225. 1952.
 HOKIN, L. E. Biochim. et Biophys. Acta, 18:379. 1955. Edited by W. D. 186

- 13. Hokin, L. E.
 14. Hokin, L. E.
 15. Hokin, L. E.
 16. Hokin, L. E.
 16. Hokin, L. E.
 17. Hokin, L. E. and Hokin, M. R.
 18. Hokin, L. E. and Hokin, M. R.
 19. Hokin, L. E. and Hokin, M. R.
 20. Hokin, L. E. and Hokin, M. R.
 21. Hokin, L. E. and Hokin, M. R.
 22. Hokin, M. R.
 23. Hokin, M. R.
 24. Hokin, M. R.
 25. Hokin, M. R.
 26. J. Biol. Chem. 1956. In press.
 27. Hokin, M. R. and Hokin, L. E.
 28. J. Biol. Chem. 203:167.
 29. 1953.
 29. Hokin, M. R. and Hokin, L. E.
 20. J. Biol. Chem. 209:549.
 20. Hokin, M. R. and Hokin, L. E.
 21. J. Biol. Chem. 209:549.
 21. Hokin, M. R. and Hokin, L. E.
 22. Hokin, M. R. and Hokin, L. E.
 23. Hokin, M. R. and Hokin, L. E.
 24. Hokin, M. R. Biochem. 203:167.
 25. 1953.
 26. Hokin, M. R. Biochem. 203:167.
 27. 1953.
 28. M. and Albert, S.
 28. M. and Righer, S.
 29. 1955.
 20. Hokin, M. R.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 1955.
 20. 195

- 23. Hokin, M. R. and Hokin, L. E. J. Biol. Chem. 203: 167. 1953.
 24. Hokin, M. R. and Hokin, L. E. J. Biol. Chem. 209: 549. 1954.
 25. Johnson, R. M. and Albert, S. Arch. Biochem. and Biophys. 35: 340. 1952.
 26. Kennedy, E. P. and Weiss, S. B. J. Am. Chem. Soc. 77: 250. 1955.
 27. Logan, J. E., Mannell, W. A., and Rossiter, R. J. Biochem. J. 51: 470. 1952.
 28. Markham, R. and Smith, J. D. Nature, 168: 406. 1951.
 29. Morton, M. E. and Schwartz, J. R. Science, 117: 103. 1953.
 30. Okuhara, E. and Nakayama, T. J. J. Biol. Chem. 215: 295. 1955.
 31. Schmidt, G. and Thannhauser, S. J. J. Biol. Chem. 161: 83. 1945.
 32. Schucher, R. and Hokin, L. E. J. Biol. Chem. 210: 551. 1954.
 33. Strickland, K. P. Can. J. Research, E, 30: 484. 1952.
 34. Taurog, A., Chaikoff, I. L., and Perlman, I. J. Biol. Chem. 145: 281. 1942.

DISCUSSION: R. J. ROSSITER¹

Dr. Hokin's paper is of great interest to the workers in our laboratory. Although we have not been concerned with pancreas tissue to any extent, brain tissue comes within our range of interest. Mr. Magee has confirmed Hokin's acetylcholine effect with slices of cat and guinea pig brain and he and Mr. McMurray have shown that phosphoinositide and phosphatidic acid are strongly labelled from inorganic P22 in both slice and homogenate preparations. With slices, in contrast to homogenates, the three phosphoglycerides are also labelled, phosphatidyl choline being the most active. To demonstrate this, use has been made of (a) the paper chromatographic procedures of Dawson (1) for the separation of phospholipid hydrolysis products, and (b) the paper chromatographic method described yesterday by Dr. Berry for the intact lipids. Dr. Hokin's paper is of great interest to the workers in our laboratory. Although we have

¹Contribution from the Department of Biochemistry, University of Western Ontario, London, Ontario.

Dr. Hokin has described conditions (presence of acetylcholine) where there is a change in the labelling of phospholipid from both inorganic P²⁵ and ethanolamine-2-C¹⁴, without a corresponding change in the labelling from glycerol-1-C¹⁴. In our laboratory, Dr. Kline, Mr. Pritchard, and Mrs. McPherson have investigated other instances where physiological factors cause a change in the labelling of phospholipid from one precursor, but not from others. factors cause a change in the labelling of phospholipid from one precursor, but not from others. Depriving a rat of food for 24 hr. causes a decrease in the subsequent labelling of the phospholipid of liver slices from acetate-1-C¹⁴. However, with glycerol-1-C¹⁴, glycine-2-C¹⁴, or inorganic P²⁸ as precursor, there is no such decrease. Exposing the animal to a cold environment causes a similar decrease in the labelling from acetate-1-C¹⁴, but not from glycerol-1-C¹⁴, glycine-2-C14, or inorganic Paz.

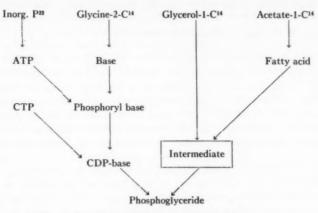


Fig. 1. Scheme for labelling phosphoglyceride.

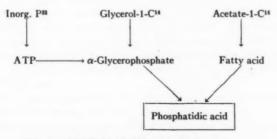


Fig. 2. Scheme for labelling phosphatidic acid.

Mr. Pritchard has shown that in experiments with liver slices, acetate-1-C¹⁴ labels only the fatty acid, glycine-2-C¹⁴ labels only the bases (serine, ethanolamine, and, to a lesser extent, choline), and glycerol-1-C¹⁴ labels only the glycerol portion of the phosphoglyceride molecule. Fig. 1 shows a scheme representing the pathway of the labelling for a typical phosphoglyceride. In this scheme, the labelling of ATP from inorganic P²⁴ and the labelling of the base from glycine-2-C¹⁴ are well-known reactions. The evidence for the labelling of phosphoryl base and for the formation of phosphoglyceride through CDP-base has been summarized most ably by Dr. Kengedy.

and for the formation of phosphoglyceride through CDT-base has been sampled ably by Dr. Kennedy.

In his paper Dr. Hokin demonstrated a change in the labelling of the phosphate-base moiety, with no change in the labelling of the fatty acid, with no change in the labelling of the glycerol, base, or beathers.

phosphate.

As pointed out by Dr. Kennedy, the scheme on Fig. 1 contains an intermediate capable of accepting base and phosphate from CDP-base. One suggestion is that this intermediate is phosphatidic acid. This would be labelled as in Fig. 2 (after Kennedy) and, indeed, many workers have claimed that phosphatidic acid is strongly labelled from inorganic P³⁸ in vitro. However, if phosphatidic acid is an immediate precursor of the phosphoglycerides, one would expect it to be more strongly labelled with glycerol-1-C¹⁴ than the phosphoglycerides themselves. Using the techniques of Dawson (1), Mr. Pritchard has shown that in liver slices respiring in the presence of glycerol-1-C¹⁴, there is good labelling of the glycerol moiety of all three phosphoglycerides, but poor labelling of the glycerol of the phosphatidic acid. In other words, after hydrolysis of the lipid extract with 0.2 N alkali for 15 min., there is poor labelling of α-glycerophosphate, presumed from phosphatidic acid, but good labelling of the α-glycerophosphate moiety of GPC, and also GPE and GPS. This would appear to eliminate phosphatidic acid as the intermediate.

1. DAWSON, R. M. C. Biochim. et Biophys. Acta, 14: 374. 1954.

THE FUNCTION OF PHOSPHOLIPIDS1

By J. M. R. BEVERIDGE

I think we all make the tacit assumption that our efforts in research will enable us to gain a better understanding of the nature of a process, or in other words, to find out how something functions. It follows, on this basis, that a knowledge of the metabolic role of phospholipids would comprise the fruition of all fundamental work on this subject.

During this symposium we have heard how the techniques of varied fields have been applied to studies on the phospholipids. Reference has been made to the methods utilized for the elucidation of their chemical structure, for their synthesis, and for their isolation. The means by which these compounds are anabolized and catabolized in the body have been discussed. One might very well assume that from this abundant store of information, authoritative conclusions might be reached regarding the function of the phospholipids. As you have probably already inferred from the manner in which that statement was introduced, we are still a long way from reaching such a happy state of affairs.

In a presentation of this nature, it is obviously impossible to consider in detail the evidence for the various functions that have been proposed for these compounds and for that reason I will restrict my consideration to a few of the better known postulated roles that have been the subject of a goodly number of experimental trials.

First of all let us dispose of the so-called structural function. It is of course well known that these substances are integral components of all cells. However, this fact certainly does not preclude a so-called metabolic function in all of the sites in which these compounds are found. The structural and the other functions to be discussed here are by no means mutually exclusive.

In the consideration of the other roles postulated for phospholipids in fat metabolism, I will take most of the time to deal with three of the main points at issue. These are:

- (i) that phospholipids act as intermediates in the resynthesis of triglycerides during the absorption of fat;
- (ii) that phospholipids comprise a vehicle for the transport of neutral fat in the form of fatty acids;
- (iii) that phospholipids facilitate the oxidation of fatty acids.

The suggestion that phospholipids act as intermediates in the synthesis of neutral fat from fatty acids within the intestinal mucosa during the absorption of fat was first advanced by Sinclair (52) in 1929. In support of his theory Professor Sinclair submitted data to show that the phospholipids undergo a rapid turnover during fat absorption as was indicated by the rapid change in

¹Manuscript received December 1, 1955.
Contribution from the Department of Biochemistry, Faculty of Medicine, Queen's University, Kingston, Ontario. This paper was presented at the Symposium on the Chemistry and Physiology of Phospholipids held at London, Ont., October 12-13, 1955.

the degree of unsaturation of the constituent fatty acids following the feeding of highly unsaturated fat. Similar findings were reported by Artom and Peretti (7) who employed iodized fatty acids as a label. Further work by Sinclair (54, 55) who utilized elaidic acid as an indicator lent additional support to his thesis and also revealed wide differences in the rate of phospholipid turnover in different tissues, the rate being relatively rapid in the intestinal mucosa, liver, and blood plasma and slow in muscle and brain. It was also thought to be of significance that although there was a rapid phospholipid turnover in the intestine even during fasting, the ingestion of a meal high in fat in contrast to carbohydrate caused a still greater rate of phospholipid turnover (8). This finding by Artom and his colleagues, was confirmed by Schmidt-Neilson (51) and by Favarger (26), who, using P³² as an indicator, demonstrated that more phospholipid was synthesized by the intestine when it was absorbing oleic acid than when it was in the resting state or when it was absorbing glucose. Verzar's work demonstrating the effect of the phospholipid precursors, glycerol and phosphate, in accelerating fat absorption also helped to make Sinclair's hypothesis an attractive one (cf. Verzar and McDougall (59)).

However, although the foregoing information is highly suggestive of a role for phospholipids in the absorption of fat, it does not provide positive proof for the hypothesis that they are in fact intermediates in the resynthesis of fat during this process and indeed subsequent investigations have lent little further weight to this postulate.

Thus work by Artom and Cornatzer (4, 5) showed that when choline was given to rats on a diet low in protein, the incorporation of P³² into the phospholipids of the intestinal mucosa was increased. This effect was enhanced when fat was given simultaneously but little or no stimulation was observed when fat was given alone. At first glance it would appear that these results provide additional support to the thesis under discussion but these authors emphasized that the increases in phospholipid turnover in the rats receiving choline and fat were still much below the range that one would expect if all of the fatty acids in the administered fat were converted into phospholipids. In similar vein was the conclusion reached by Zilversmit, Entenman, and Chaikoff (62) who found that in the dog there was no increase in phospholipid turnover in the intestinal mucosa during the absorption of a fatty meal and that in the rat such increases as might occur were too small to account for all of the absorbed fat having passed through the phospholipid stage.

The point that should be immediately raised here, of course, is the possibility that dietary fat need not be completely hydrolyzed prior to absorption. The theory that much of the ingested fat may be absorbed in the form of fine droplets of triglycerides was extant and widely held in the last century (cf. Leathes and Raper (38)) and recently this suggestion has been revived and modified by Frazer (28) who claims that the process of emulsification accounts for the absorption of something of the order of 50% of the fat, the remainder being absorbed in the form of fatty acids and mono- and di-glycerides. In

1952 Mattson, Benedict, Martin, and Beck (41) demonstrated that the latter two types of compounds were at least formed in the intestinal lumen during the digestion of fat. They analyzed the intestinal contents of rats following the feeding of triglyceride and found that mono- and di-glycerides accounted for 16 and 36% respectively of the total lipids. About this same time Reiser et al. (47) by feeding two types of triglyceride, one labelled in both the glycerol and fatty acid moieties by C14 and conjugated linoleic acid respectively, and the other distinguished by the fact that it was a saturated triglyceride, showed that about 25–45% of the triglycerides were completely hydrolyzed and the remainder broken down to monoglycerides and absorbed as such. In the light of these experiments, therefore, the point made by Artom et al. (4, 5) and Zilversmit et al. (62) loses at least some of its weight and importance.

An additional circumstance to be considered is the possibility that one of the components of the phospholipid fraction has a very much higher rate of turnover than that of the total mixture. This feature was mentioned in a footnote to a paper published by Pihl and Bloch in 1950 (45) but discounted by them with the statement that no evidence existed at that time to support this assumption. However, a review of the literature before and since that paper was published has revealed a number of reports showing that different phospholipids do in fact have widely different turnover rates (6, 19, 20, 32, 64, 22). Hevesy and Hahn (30) emphasize this feature and point to the marked differences noted by them in the rate of turnover of lecithin and cephalin in the liver. These workers also concluded that cephalin itself is comprised of at least two components which they termed as the "fast" and "slow" cephalin fractions. Whether or not the magnitude of the differences would be sufficient to negate the points made by Artom et al. (4, 5) and by Zilversmit et al. (62) is a question that cannot be answered categorically at this time.

In addition to the foregoing considerations it is difficult to reconcile the report by Zilversmit *et al.* (62) with that of Bollman, Flock, Cain, and Grindlay (15) who found that the feeding of fat to dogs caused up to a threefold increase in the phospholipid concentration of intestinal and thoracic duct lymph. These workers concluded that the small intestine is normally a source of phospholipids for the plasma during the absorption of fat. In any event the considerable increase in lymph phospholipid concentration noted by them presumably was due to an increased intestinal synthesis, a conclusion that is in direct conflict with the one reached by Zilversmit *et al.* who found no increase in phospholipid turnover rate in the intestine of dogs following a fatty meal (62).

A further refutation of the possibility that phospholipids act as intermediates in the resynthesis of triglycerides during fat absorption appeared in a recent publication by Reiser and Dieckert (48). These workers fed rats pure triglycerides labeled in both the glycerol and fatty acid moieties. Since the phospholipids of the intestinal mucosa and lymph had lower relative specific activity than the triglycerides they concluded that rather than the mucosal

phospholipids being the precursors of the resynthesized triglycerides, the reverse was true. Again it is unfortunate that these investigators lumped together all of the types of phospholipids since the possibility still remains that one fraction might have a much higher specific activity than the others and thus might possibly be, in theory at least, the precursor for the triglycerides synthesized during fat absorption.

In summing up the evidence for phospholipids playing the role first postulated for them by Sinclair in 1929 (52) it would appear that there is very little direct important evidence in its support and that some rather significant data have been published that militates strongly against the acceptance of this theory. However, this does not necessarily mean that phospholipids have no role to play in the digestion and absorption of triglycerides. Indeed much of the work to which we have already referred is entirely consonant with and furthermore supports the belief that phospholipids are in some way involved in these processes. To recapitulate, consider for example the following partial list of highly suggestive points in this connection: firstly, that fatty acids of dietary triglycerides are rapidly incorporated into phospholipids of the intestinal mucosa; secondly, that phospholipid turnover in the intestinal mucosa, as determined by the uptake of P³², is stimulated by the simultaneous administration of fat but not to the same extent by carbohydrate; thirdly, that choline fed to rats on low protein diets stimulates phospholipid turnover and this effect is enhanced by the simultaneous administration of fat; and fourthly that phospholipid concentrations increase in both intestinal lymph and plasma during the absorption of triglycerides. Taken together these experimental findings are strongly indicative of some role for phospholipids in fat absorption. It remains a subject for future investigations to delineate the nature of this role.

The second important function I have listed for phospholipids is their postulated action as transport agents for neutral fat in the form of fatty acids. Ever since this suggestion was made, fairly early in this century, very little in the way of supportive experimental data has been forthcoming and much of a negative nature has been published (cf. 43, 60). Indeed important and conclusive evidence has been presented in a series of papers by Chaikoff's group that militates against the acceptance of this theory. For example, Fishler, Entenman, Montgomery, and Chaikoff (27) studied the phospholipid turnover in the hepatectomized dog fed radioactive phosphate and found practically no labeled phospholipid in the plasma despite the fact that amounts comparable to those found in normal dogs appeared in the kidneys and intestines. On the basis of these results, they concluded that the liver is the main source of plasma phospholipids. In a later paper, Entenman, Chaikoff, and Zilversmit (24) demonstrated by measuring the rate of disappearance of tagged phospholipids in the plasma of normal dogs and in dogs whose liver had been by-passed by the blood circulation, that the liver was also mainly responsible for the removal of phospholipids from the plasma. The fact that the liver is the prime organ in both synthesis and removal of phospholipids

from the plasma would appear to preclude any role for these compounds in the transport of neutral fat in the form of fatty acids to the tissues throughout the body for deposition and catabolism. Further evidence concerning this point has been submitted by Goldman, Chaikoff, Reinhardt, Entenman, and Dauben (29). These workers administered labeled palmitic acid to hepatectomized dogs and found that although heart, skeletal muscle, kidney, and small intestine incorporated this fatty acid into phospholipids, essentially none was found in the plasma. It was concluded that the phospholipids synthesized by these tissues "are not normally concerned with the transport of fatty acids from one organ to another". Another experiment bearing on this problem has been published recently also from Chaikoff's laboratory (12) showing that when C14 carboxyl labeled palmitic acid was absorbed via the gut into the lymph, 96% of it was present in forms other than phospholipid. In the light of these findings there appears to be little justification for clinging to the belief that phospholipids may act as vehicular agents for fatty acids in plasma or lymph.

However, this should not be taken to mean that phospholipids have no part to play in the transport of neutral fat. The well-established finding of Barrett, Best, and Ridout (11) and Stetten et al. (56, 57) that choline promotes the mobilization of fat from the liver to the fat depots coupled with the fact that choline stimulates synthesis of phospholipids by the liver is strongly suggestive of a role for these compounds in fat transport. Macheboeuf and others (cf. reviews by Macheboeuf (40) and Oncely and Gurd (42)) have shown that plasma lipids occur almost entirely as alpha and beta lipoprotein complexes and that phospholipids are integral constituents of these substances. Iones, Gofman, et al. (33) have reported a whole series of lipoproteins of varying density, presumably beta lipoprotein, in which increasing triglyceride content goes hand in hand with decreasing density. It is also of interest in this regard to recall that Boyd (17) and Ahrens and Kunkel (1) have reported that in the presence of high lipid levels there may be no lactescence in the plasma so long as phospholipid concentrations are comparably increased and that in the face of relatively low phospholipid levels, especially if the other components are elevated, and neutral fat is of greatest significance in this regard, the plasma assumes a turbid or milky appearance. The precise relationship between the chylomicrons which are responsible for the lactescence and the two main types of lipoproteins, alpha and beta, is not clear at this time.

Of some practical significance is the report by several groups of workers (cf. Katz and Stamler (34)) that the ratio of cholesterol to phospholipid in the plasma is increased in proved cases of coronary atherosclerosis compared to individuals of the same sex, age, and weight without overt signs of this disease. A most remarkable feature of the relationship between these two lipids, and one that has long occasioned a great deal of speculation, is the relative constancy of the one to the other. The significance of this circumstance is not known but one might postulate the production of certain lipoproteins by the liver, and since the composition of these are presumably fixed

with respect to phospholipid and cholesterol, the ratios of these two would not readily change unless there was a gross change in the proportions of the various lipoproteins that were being synthesized. Thus, for example, of the two main classes of lipoproteins, alpha and beta, Russ, Eder, and Barr (50) report that the cholesterol/phospholipid ratios are of the order of 0.5 and 1.3 respectively. According to these workers (10) the beta lipoprotein fraction is increased in survivors of a myocardial infarction. This would, of course, lead to an increase in the total plasma cholesterol/phospholipid ratio. Undoubtedly, other relationships of great importance may be brought to light by further analytical studies to determine the precise nature and level of the various types of phospholipids present in the lipoprotein fractions, structures that are obviously concerned with fat transport.

Finally there remains the theory that phospholipids facilitate the oxidation of fatty acids. Perhaps the earliest reference to this possibility was made in 1891 by Loew (39) who stated that lecithin was a machine for burning fats. In a paper published about 20 years later Leathes (37) and also Dr. Bloor (13) expressed a somewhat similar belief. However, for many years there was little concrete supportive evidence presented and only during the past decade or two have any convincing data been obtained in support of the thesis.

Artom (2) in a discussion of the mechanism of the lipotropic action of choline pointed out that if choline does not increase the rate of mobilization of fat from the liver as fatty acids in the form of plasma phospholipids, "one is inclined to ascribe the lipotropic effect of this substance to an increased catabolism of fatty acids in the liver itself". The same worker (3) subsequently studied the rate at which various liver preparations, from rats fed different diets, oxidized labeled stearate or palmitate. Liver tissue from rats on a low protein diet containing added guanidinoacetic acid, given to deplete further the animal's stores of labile methyl groups, was less effective in oxidizing the fatty acid substrates than liver tissue from rats on a stock diet. The ability to oxidize these fatty acids at a normal rate was generally restored by supplementation of the low protein diet with choline, or by injection of massive doses of choline shortly before removal of the liver. Neither the addition in vitro of choline nor any of the close derivatives, betaine, betaine aldehyde, or phosphorylcholine, was effective in stimulating the oxidation of the fatty acids no matter what diet the animal had eaten prior to being killed. On the basis of these findings, Artom suggested that the lipotropic effect of choline might be due largely to the increase in the rate of fatty acid oxidation in the liver because of the action of certain substances, probably phospholipids, formed from choline in vivo.

It should perhaps be pointed out that several groups of workers have demonstrated that choline increases the turnover rate of liver phospholipids in vivo in choline-deficient rats or dogs (44, 16, 63) a circumstance that would lend weight to the foregoing suggestion. The report by Eperjessy and Zathureczky (25) that the addition of choline to rat liver slices depressed the respiratory quotient thus apparently stimulating fat oxidation and/or

depressing carbohydrate oxidation would apparently be in keeping with the thesis suggested by Artom. In a paper just published, Rodbell and Hanahan (49) reported that the addition of lecithin and certain of its derivatives to liver mitochondrial systems caused a larger stimulation of oxygen uptake than could be expected from complete oxidation of these compounds. They suggested that the phenomenon observed by them was due to "increased oxygen uptake by fatty acid derivatives".

Further evidence in accord with a role for phospholipids in fat oxidation has been reported by Zilversmit and DiLuzio (61). These authors proceeded on the assumption that since fat metabolism is going on rapidly in the diabetic dog, phospholipid synthesis should be increased if these compounds are involved in fat oxidation. They found that phospholipid synthesis was in fact markedly elevated in the liver and increases of smaller magnitude were noted in the small intestine and kidney. The same workers (23) found an increased turnover rate for liver and plasma phospholipids in phlorizinized dogs. It was concluded that the data were compatible with the hypothesis under discussion.

Of possible significance in regard to this section is the finding of a number of workers (9, 21, 18, 35) that mitochondria, which contain the enzyme systems for many oxidation processes including fatty acid oxidation, are very high in phospholipid content. Also pertinent is the report of Kielley and Meyerhof (36), who concluded that lecithin is an essential constituent of the adenosine triphosphatase obtained from muscle, and that of Swanson and Mitchell (58) who found "very considerable amounts of phospholipids in a highly purified preparation of adenosine triphosphatase separated from the nucleoprotein of liver nuclei". And finally it is of interest to remark on the paper by Quastel and Braganca (46) who noted that although lecithinase A does not affect a large number of soluble enzymes, it does inactivate all the enzyme systems located chiefly or only in the granular fractions of the cytoplasm.

The foregoing investigations would appear to constitute a fair body of evidence in support of the theory that phospholipids play some role in facilitating fat oxidation.

It would not be profitable in the time left at my disposal to attempt a discussion of other postulated roles for phospholipids. I would be remiss, however, if all reference to the lipid component of thromboplastin were omitted. Following the original work by Howell and McLean (cf. Howell (31)) early in this century it has been assumed, and many subsequent workers in this field are still in agreement with the belief, that the thromboplastic lipid is "cephalin". Certainly there is no doubt of the reality of the greatly accelerating effect of certain phospholipid preparations, when tested at appropriate concentrations, on the conversion of prothrombin to thrombin. However, as Wittcoff (60) has indicated in his excellent monograph on the phosphatides, there exists a great deal of uncertainty regarding the identity of the phospholipid in question and as matters now stand it is impossible to

state authoritatively (a) what the nature of the lipid is that might be associated with thromboplastin and (b) whether or not a lipid is in fact an essential component of this substance.

In a similar way the function of phospholipids in brain and nerve must be passed over. Although the particularly high level of phospholipids in these tissues has long been known their function is still enshrouded in mystery. It is most remarkable that despite the probable role of phospholipids in promoting fat oxidation, the brain, with its abundance of these compounds, does not utilize fat as a source of energy.

There are two intriguing experimental findings to which I feel attention should be drawn. One of these concerns the rather considerable work of Bloor and his colleagues on the direct parallel between physiological activity and phospholipid content of a tissue (cf. Bloor (14)). The other is the interesting report by Sinclair (cf. Sinclair (53)) that although unsaturated fatty acids entered very rapidly into the phospholipid molecule they were not given up nearly so readily from these compounds. It seems to me that both these circumstances are of great significance and a logical explanation of them would do much to clarify the role of phospholipids in metabolism.

To sum up briefly the three postulated functions for phospholipids that we have discussed, it would appear that although there is little to support the thesis that phospholipids act as intermediates in the resynthesis of triglycerides during the absorption of fat, there are good grounds for believing that they are in some way involved in this process. Cogent evidence has been obtained militating against the acceptance of the belief that phospholipids comprise a vehicle for the transport of fatty acids. In this connection there would appear to be excellent reasons for assigning to β -lipoprotein, of which phospholipids are integral constituents, an important role in the transport of neutral fat. And finally there has been presented sound evidence in support of the theory that choline-containing phospholipids, probably lecithins, facilitate the oxidation of fatty acids.

References

- Анкеns, E. H. and Kunkel, H. G. J. Exptl. Med. 90: 409. 1949.
 Актом, С. Phosphorus metabolism. Vol. 2. Edited by McElroy and Bently Glass. The Johns Hopkins Press, Baltimore, Md. 1952. p. 203.
- 3. Artom, C. J. Biol. Chem. 205: 101. 1953.
 4. Artom, C. and Cornatzer, W. E. J. Biol. Chem. 165: 393. 1946.
 5. Artom, C. and Cornatzer, W. E. J. Biol. Chem. 171: 779. 1947.
 6. Artom, C. and Freeman, J. A. J. Biol. Chem. 135: 59. 1940.

- 7. ARTOM, C. and PERETTI, G. Arch. intern. physiol. 42:61. 1935-36.
- 8. ARTOM, C., SEGRÉ, E., and SARZANA, G. Arch. intern. physiol. 47: 245. 1938.
- 9. Ball, E. G. and Cooper, O. J. Biol. Chem. 180: 113. 1949.
- 10. BARR, D., RUSS, E., and EDER, H. Am. J. Med. 11: 480. 1951.
- 11. BARRETT, H. M., BEST, C. H., and RIDOUT, J. H. J. Physiol. 93: 367. 1936.
- 12. BLOOM, B., CHAIKOFF, I. L., REINHARDT, W. O., and DAUBEN, W. G. J. Biol. Chem. 189: 261. 1951.

 13. Bloor, W. R. J. Biol. Chem. 24: 447. 1916.

 14. Bloor, W. R. Biochemistry of the fatty acids. Reinhold Publishing Corporation, New
- York. 1943.

 BOLLMAN, J. L., FLOCK, E. V., CAIN, J. C., and GRINDLAY, J. H. Am. J. Physiol. 163: 41. 1950. 41.

BOXER, G. E. and STETTEN, D., JR. J. Biol. Chem. 153: 617. 1944.
 BOYD, E. M. Trans. Roy. Soc. Can. V, 31: 11. 1937.

18. CHANTRENNE, H. Biochim. et Biophys. Acta, 1:437.

CHARGAFF, E. J. Biol. Chem. 128: 587. 1939.
 CHARGAFF, E., OLSON, K. B., and PARTINGTON, P. F. J. Biol. Chem. 134: 505. 1940.

CLAUDE, A. J. Exptl. Med. 84: 61. 1946.
 DAWSON, R. M. C. Biochem. J. 57: xv. 1954.

23. DILUZIO, N. R. and ZILVERSMIT, D. B. Am. J. Physiol. 170: 472. 1952.

24. Entenman, C., Chaikoff, I. L., and Zilversmit, D. B. J. Biol. Chem. 166: 15. 1946. 25. Eperjessy, A. and Zathureczky, Z. V. Hoppe-Seyler's Z. physiol. Chem. 282: 80. 1944.

FAVARGER, P. Helv. Physiol. et Pharmacol. Acta, 7: C-41. 1949.
 FISHLER, M. C., ENTENMAN, C., MONTGOMERY, M. L., and CHAIKOFF, I. L. J. Biol. Chem. 150: 47. 1943.

- Chem. 150; 47, 1945.

 28. Frazer, A. C. Physiol. Revs. 26: 103. 1946.

 29. Goldman, D. S., Chaikoff, I. L., Reinhardt, W. O., Entenman, C., and Dauben, W. G. J. Biol. Chem. 184: 727. 1950.

 30. Hevesy, G. and Hahn, L. Kgl. Danske Videnskab. Selskab Biol. Medd. 15: 5. 1940.

 31. Howell, W. H. Physiol. Revs. 15: 435. 1935.

 32. Hunter, F. E. Proc. Soc. Exptl. Biol. Med. 46: 281. 1941.

- Jones, H. B., Gofman, J. W., Lindgren, F. T., Lyon, T. P., Graham, D. M., Strisower, B., and Nichols, A. V. Am. J. Med. 11: 358. 1951.
 Katz, L. N. and Stamler, J. Experimental atherosclerosis. Charles C. Thomas, Publisher, Springfield, Ill. 1953.
- KENNEDY, E. P. and LEHNINGER, A. L. J. Biol. Chem. 179: 957. 1949.
 KIELLEY, W. W. and MEYERHOF, O. J. Biol. Chem. 183: 391. 1950.

37. LEATHES, J. B. Lancet, 1:593. 1909.

38. Leathes, J. B. and Raper, H. S. The fats. 2nd ed. Longmans, Green & Co., Ltd., London. 1925. London.

39. LOEW, O. Biol. Zentr. 11: 269. 1891.

40. Macheboeuf, M. Blood cells and plazma proteins. Edited by J. L. Tullis. Academic Press, Inc., New York. 1953. Sect. VII, Chap. 2.

41. MATTSON, F. H., BENEDICT, J. H., MARTIN, J. B., and BECK, L. W. J. Nutrition, 48: 335. 1952.

 ONCLEY, J. L. and GURD, F. R. N. Blood cells and plasma proteins. Edited by J. L. Tullis. Academic Press, Inc., New York. 1953. Sect. VII, Chap. 1. 43. PAGE, I. H. The chemistry of the brain. Charles C. Thomas, Publisher, Springfield, Ill.

1937.

 PERLMAN, I. and CHAIKOFF, I. L. J. Biol. Chem. 1
 PIHL, A. and BLOCH, K. J. Biol. Chem. 183: 431. J. Biol. Chem. 127: 211. 1939.

1950.

46. QUASTEL, J. H. and BRAGANCA, B. M. Federation Proc. 11: 272. 1952.

47. REISER, R., BRYSON, M. J., CARR, M. J., and KUIKEN, K. A. J. Biol. Chem. 194: 131. 1952.

48. Reiser, R. and Dieckert, J. W. Proc. Soc. Exptl. Biol. Med. 87: 622. 1954.

 RODBELL, M. and HANAHAN, D. J. J. Biol. Chem. 214: 595. 1955.
 RUSS, E., EDER, H., and BARR, D. Am. J. Med. 11: 468. 1951. 51. SCHMIDT-NEILSEN, K. Acta. Physiol. Scand. Suppl. 37:12. 1946.

52. SINCLAIR, R. G. J. Biol. Chem. 82:117. 1929. 53. SINCLAIR, R. G. Physiol. Revs. 14: 351. 1934. SINCLAIR, R. G. J. Biol. Chem. 134: 83. 1940.
 SINCLAIR, R. G. J. Biol. Chem. 134: 89. 1940.

56. STETTEN, D., JR. and GRAIL, G. F. J. Biol. Chem. 148: 509. 1943.

 STETTEN, D., JR. and SALCEDO, J., JR. J. Biol. Chem. 156: 27. 1944.
 SWANSON, M. A. and MITCHELL, M. C. Federation Proc. 11: 296. 1952.
 VERZAR, F. and McDougall, E. J. Absorption from the intestine. Longmans, Green & Co., Inc., New York. 1936.

60. WITTCOFF, H. The phosphatides. Reinhold Publishing Corporation, New York. 1951.

61. ZILVERSMIT, D. B. and DILUZIO, N. R. J. Biol. Chem. 194: 673. 1952. 62. ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. J. Biol. Chem. 172: 637. 1948. ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. J. Biol. Chem. 176: 193. 1948.
 ZILVERSMIT, D. B., ENTENMAN, C., and CHAIKOFF, I. L. J. Biol. Chem. 176: 209. 1948.

DISCUSSION: ORVILLE F. DENSTEDT1

I should like to elaborate upon one role of the phospholipids that the preceding speaker had time merely to touch upon, namely, their 'structural' or 'physicochemical' role. The ubiquitous distribution of the phospholipids in plant and animal tissues led workers as far back as 75 years ago, to surmise that these substances must play a fundamental role in living cells. Mayer and Schaeffer (15), in 1914, observed that a residual amount of fatty acid persisted in the tissues of animals even at death from starvation. Their findings suggested, furthermore, that in starvation the intracellular phospholipids that make up this irreducible minimum can be forced to yield one of their two fatty acid components. Referring to these observations Leathes (13) has commented: "A picture is presented as it were of the emaciated figure of the phospholipids persisting at their post in the starving cell an immovable part of its protoplasm".

The apparent essentiality of the phospholipids in the structure and metabolism of the cell prompted many workers during the past 50 years to examine the lipids of normal and pathological tissue. Unfortunately, owing to the inadequacies of the methods and the unawareness of the existence of certain of the lipids most of the data collected in former years have little more than a qualitative significance. With the discovery of the serine phosphatides by Folch (6), and of the inositol phosphatides by Folch and Woolley (7) in 1942, the accuracy of the values previously reported for the phospholipids and particularly for the 'cephalin'

content of tissues became doubtful.

The remarkable advances of the past 10 years, in our knowledge of the enzymology and metabolism of the various body tissues have necessitated the study of the distribution of the lipids within the cell itself. The examination of the finer structures of the cell has been aided, and indeed made possible, by the introduction of phase-polarized light, and electron microscopy and revolutionary new techniques such as ultracentrifugation, electrophoresis, chromatography, and spectrophotometry. We now know that all the solid components of the cells—the plasma membrane, nucleus, nucleolus, golgi apparatus, mitochondria down to the microsomes—contain phospholipid associated with cholesterol, nucleoprotein, and other materials (1, 2). The mitochondria of animal cells and the chloroplasts of plant cells contain about 35% of lipids. In the mitochondria of animal cells phospholipid makes up about 4%, cholesterol 2%, and fat 28% of the total lipid. Ribonucleic acid is present in the cytoplasmic particles, and possibly in small amounts in the nucleus, while desoxyribonucleoprotein is confined to the nucleus.

Little is known about the manner in which the phospholipids are distributed among the various solid components of the cell. The evidence from studies (25, 16, 22) on the birefringence of the plasma membrane and the ground cytoplasm indicates that the lipids are closely associated with protein and that the lipid molecules are oriented radially on the nucleoprotein fibers and at right angles to the long axis. The prevailing view appears to be that the lipids of the membranes are arranged in a uni- or bi-molecular layer on a layer of protein (25, 26), but there is evidence also to support the view that the lipids may be systematically dispersed in an aqueous phase within a network of protein fibers (17). Some of the phospholipid in the envelope of the erythrocyte appears to be loosely bound since it can be removed by repeated washing with saline, apparently without greatly altering the stability of the membrane (14).

There is strong evidence that the phospho- and other lipids play a fundamental role in the semipermeability of the cell membrane (4, 5, 10, 11, 18). The membrane has been likened to a sieve with pores that open and close rhythmically, permitting the passage of certain substances and excluding others. Whatever the mechanism may be, its operation is controlled by the metabolic activity of the cell. Furthermore, the passage of certain ions and other substances is effected by an active transfer mechanism requiring the expenditure of energy while the passage of certain other ions and substances appears to occur by passive diffusion.

while the passage of certain other ions and substances appears to occur by passive diffusion. The nucleus and smaller inclusion bodies, possibly including some of the submicroscopic particles, also have semipermeable limiting membranes. Some authorities have pictured the ultimate reticulum of the ground cytoplasm as being made up of bundles of ribonucleoprotein fibers surrounded by a film of radially oriented lipid molecules (8, 22, 23, 24). Electronmicrographs recently obtained by Porter (19) and others indicate the existence of more complex submicroscopic structures made up of fenestrated membranous elements in the form of flattened, elongated vesicles. The make-up of the ground cytoplasm has been discussed by Frey-Wyssling (8), Claude (1, 2), and many others, and the various modern techniques for examining the fine elements of the cell have been described by Schmitt (21).

Consideration of the morphology of the cell impresses one with the diversity of the types

Consideration of the morphology of the cell impresses one with the diversity of the types of surface presented and the enormity of the combined areas of the interfaces of the cytoplasm. Analogous surfaces presumably exist also within the solid bodies of the cell. At these lipid-water interfaces the enzyme-catalyzed chemical reactions of metabolism take place. "Life resides at the interface". Furthermore, the high degree of organization of the surfaces is maintained through a continuous expenditure of energy by the cell. Just as the highly

¹Contribution from the Department of Biochemistry, McGill University, Montreal, Quebec.

accomplished musician must practise several hours daily to maintain the skill he has already acquired, so the cell is obliged to spend energy to maintain its state of organization and efficiency. In the latter case maintenance is achieved through 'turnover'—the continuous breaking down and replacement of highly organized structures and surfaces. "The mystery of life is hidden in activity." (Bunge).

The role of the surfaces in relation to metabolic processes has been discussed by Danielli

and Davies (3) and others (9, 20, 24)

The significance of the phospholipids as integral components of the interfaces becomes even more impressive when one recalls their strategic association with nucleoprotein and their possible function as an organized reservoir of phosphoric acid and as intermediaries in the possible full color as an organized reservoir of phosphoric action and as in the internation of fatty acids. In the intestinal mucosa they may serve also as agents for the dispersal of fat for transport as such in the lymph and the blood. Some of the fatty acids are required for the replenishment of phospholipids, both free and attached at the interfaces. Similarly, in the utilization of fatty acids in the cell some molecules may be used in the synthesis of phospholipid while others may be oxidized. This dual involvement of the phospholipids may help to explain some of the quantitative discrepancies referred to by the preceding speaker.

At times, when the metabolic activity is stepped up and mitochondria and other particles are being multiplied or replenished, more phospholipid may be required both for the metabolic and the physicochemical purposes. In circumstances of diminished activity, on the contrary, owing to prolonged exposure to cold, chronic illness, or aging, the rate of turnover diminishes and retrograde changes occur at the interfaces. In the degeneration of tissues the lipids are released from lipoproteins. The highly dispersed and organized form that characterizes the normal tissue breaks down and the lipids coalesce and separate out (12).

Phospholipids, particularly the lecithins, are surface-active agents. They appear to be intimately connected with the property of irritability in living cells and in some of the more complex viruses. In this action the phospholipids may require the association of Ca++ and other ions. The circumstance that the enzyme ATP-ase contains phospholipid in its make-up and that this enzyme is activated by Ca⁺⁺ ions also may have significance. The more one contemplates the so-called metabolic and physicochemical involvements of the phospholipids the less the distinction between the two roles becomes.

CLAUDE, A. Biol. Symposia, 10:111. 1943,
 CLAUDE, A. Ann. N.Y. Acad. Sci. 58: 854. 1950.
 DANIELLI, J. F. and DAVIES, J. T. Advances in Enzymol. 11:35. 1951.
 DAYSON, H. and DANIELLI, J. F. Permeability of natural membranes. Cambridge University Press, London. 1943.

- DAYSON, H. and DANIELLI, J. F. Permeability of natural membranes. Cambridge University Press, London. 1943.
 DAYSON, H. and DANIELLI, J. F. Permeability of natural membranes. The MacMillan Co., New York. 1943.
 FOLCH, J. J. Biol. Chem. 146: 34. 1942.
 FOLCH, J. and WOOLLEY, D. W. J. Biol. Chem. 142: 963. 1942.
 FREY-WISSLING, A. Submicroscopic morphology of protoplasm and its derivatives. Translated by J. J. HOGEBOOM, G. H. and Kuff, E. L. Federation Proc. 14: 633. 1955.
 JACOSS, M. H. Ann. N.Y. Acad. Sci. 50: 824. 1950.
 JACOSS, M. H. Modern trends in physiology and biochemistry. Edited by E. S. G. Barron. Academic Press, Inc., New York. 1952. p. 149.
 JOINSON, A. C., MCNABR, A. R., and ROSSITER, R. J. Biochem. J. 45: 500. 1949.
 JEATHES, J. B. and RAPER, H. S. Monographs on biochemistry. Longmans, Green and Co., Ltd., London. 1925.
 JONESON, E. BIOCHEM, J. 60: 692. 1955.

Beathes, J. B. and Raper, H. S. Monographs on biochemistry. Longmans, Green and Co., Etc., London. 1925.
 Lovelock, J. E. Biochem. J. 60: 692. 1955.
 Loverer, L. Advances in Enzymol. 81: 1. 1948.
 Mayer, H. and Schafferr, G. J. physiol. et pathol. gen. 16: 204. 1914.
 Monne, L. Advances in Enzymol. 81: 1. 1948.
 Parrar, A. K. and Ballentine, A. Modern trends in physiology and biochemistry. Edited by E. S. G. Barron. Academic Press, Inc., New York. 1952. p. 77.
 Ponder, E. Hemolysis and related phenomena. Grevele and Stratton, New York. 1948.
 PORTER, K. Federation Proc. 14: 673. 1955.
 Runnstrom, J. Modern trends in physiology and biochemistry. Edited by E. S. G. Barron. Academic Press, Inc., New York. 1952. p. 47.
 SCHMITT, F. O. Medical physics. Edited by O. Glasser. Year Bk. Pubs. Inc., Chicago. 1944. p. 1586.
 SCHMITT, F. O. Medical physics. Edited by O. Glasser. Year Bk. Pubs. Inc., Chicago. 1944. p. 1586.
 SCHMITT, F. O. Advances in Enzymol. 7: 35. 1947.
 SCHMITT, F. O. Advances in Enzymol. 7: 35. 1947.
 SWANN, M. M. and MITCHISON, J. M. Progress in biophysics, Vol. 2. Edited by Butler and Randall. Pergamon Press Ltd., London. 1951. p. 1.
 WAUGH, D. F. Ann. N.Y. Acad. Sci. 30: 835. 1950.

Symposium on Neurophysiology

This symposium was held October 15, 1955, during the general session of the 19th Annual Meeting of the Canadian Physiological Society, in London, Ontario. Dr. Wilder Penfield of McGill University, Montreal, Que., acted as Chairman of the Symposium, which was organized by Dr. G. W. Stavraky of the University of Western Ontario, London, Ontario.

THE STRUCTURAL BASIS OF SOME CORTICODIENCEPHALIC RELATIONS:

By J. AUER

Since the important contributions to our knowledge of thalamocortical relations by Walker (32) considerable progress has been made with anatomical, physiological, and clinical methods towards an understanding of the relations between diencephalon and cortex. One might say that Walker's systematic analysis more or less paved the way towards a new approach. I am grateful to have a few minutes at this meeting to analyze from the structural point of view a thesis about the corticodiencephalic relations which we owe in principle to the physiologists. This thesis may be formulated in different ways but I should like to choose the following question as a simplified approach to any morphological study. What is the structural foundation of the functional (14) and clinical (19) data which have led to the assumption that the diencephalon contains differentiations with an integrative function in corticodiencephalic neural mechanisms controlling activity in general and awareness in particular?

Before going into details the physiological methods which have revealed the data relative to this thesis should be mentioned. Strychnine neuronography as well as multiple recording, in the brain stem, of electrical potentials resulting from stimulation of the cortex and striatum (and vice versa) (17, 10, 26) has been widely used in different institutions, and the anatomist must admit that he is often at a loss to find a substratum for connections inferred from these studies. It seems to be well accepted at present that spikes recorded in the brain stem do give evidence of the course of axons from the area of strychninization. The question of termination of such axons would appear to be a difficult problem to solve, particularly in areas of unbundled passage, as is the case with practically the whole reticular formation. A similar difficulty is encountered in the recording of the electrical potentials, where moreover the questions of transmission in the synapses and the size of the axons play a role. While the rate of stimulation and the strength of the stimulus serve to some extent as a control, it seems reasonable to assume that the size and length of the neuron and also the number of cells stimulated are often beyond control. Much is to be expected, therefore, from stimulation of single cells with microelectrodes.

¹Manuscript received December 13, 1955.

Contribution from the Department of Anatomy, University of Ottawa, Ottawa, Ontario. This paper was presented at the Symposium on Neurophysiology held as part of the Annual Meeting of the Canadian Physiological Society, London, Ontario, October 13-15, 1955. Supported by a grant from the National Research Council, Canada.

As I hope to explain in the course of this presentation, the most reliable method of observing neurons and their course in the central nervous system is still the silver impregnation method with all its modifications, some more suitable than others. Neuronal networks as found in the diencephalon and mesencephalon do not lend themselves easily to observation in normal material and, therefore, experimental material is now most frequently used. Nevertheless, the beautiful work of Bodian (3) in the opossum may still be considered an outstanding contribution to our knowledge of normal material. The experimental approach allows the study of lesions and their consequences away from the diencephalon, for example in the cortex or in different regions of the striatum. Secondary degeneration of the axons and terminals may then be followed with varying success with any of the silver strains described in the literature (24). The fibers break up and show swelling and beading while the terminals undergo various changes which are all centered in and around a ring-like structure described as the terminal bouton. Unfortunately, these terminal boutons are of different size and form in different areas of the central nervous system. Moreover, Young (34) has recently proved that all techniques used so far only demonstrate a small percentage of the endings in any case. He, therefore, has gone over to an entirely new method of demonstrating terminals, i.e. with low power electron microscopy. This technique has already shown that terminals are never without some kind of sheath and that there is little space devoid of boutons on the perikaryon and dendrites. This approach will definitely open up new fields in this kind of research. Another method employed with good results, particularly in bundled axons, is now used by Verhaart (30) and his staff. This is the Häggqvist method, which stains normal axons blue and their sheath bright red. Degenerating fibers fail to take the blue color. Again the difficulty here is to follow scattered fibers. However, this method is a very suitable one for counting abnormal and normal axons in bundles.

Taking these restrictions into consideration, several observations on the structure of the diencephalon and its reciprocal connections with other regions have now been accepted. In order to explain those that have some importance in our discussion, I must restrict myself somewhat and, therefore, I shall mainly deal with those parts of the diencephalon known as thalamus, hypothalamus, and subthalamus. Nissl studies of these regions show that the thalamus has a cellular configuration of much greater complexity than the two other parts, which are more fibrous in nature. The elements to be discussed from the point of view of cellular and fibrous structures in these regions are generally referred to as the diencephalic reticular formation, consisting of the posterior part of the hypothalamus, the thalamic midline, intralaminar and reticular nuclei as well as the greatest part of the subthalamus. The arrangement of these cells has recently been analyzed by Fortuyn (5) from a developmental viewpoint. One can observe in these unspecific nuclei a most intricate network of fibers which on the basis of earlier studies, on experimental (1) as well as normal material, are no doubt characterized by short neuronal connections. It is a matter of discussion which long fibers leave this multineuronal region and which fibers enter it. The first class of fibers, efferent fibers, have been studied anatomically with the method of retrograde degeneration. After Walker (32), the observations of Powell (21) and Rose (25) should particularly be mentioned. It is now clear that the nucleus reticularis projects upon the cortex and that the topographical distribution of these efferents corresponds with similar distributions in the principal thalamic nuclei as revealed by Walker (32). The midline nuclei, namely the nucleus rhomboidalis reuniens and paraventricularis, project upon the limbic cortex according to Powell (21). This author confirmed the findings of Fortuyn (5) that there is no direct efferent connection from the intralaminar nuclei to the cortex. On the contrary, these nuclei seem to be connected directly with the striatum. The centrum medianum seems to possess a topological distribution of its efferents in the caudate, according to Powell (21).

Both cortex and striatum are therefore involved in receiving axons from the so-called unspecific thalamic nuclei. This evidence conflicts in part with physiological data (29). The principal nuclei, namely, the dorsomedial, anterior, ventral, lateral, and posterior groups, are now well known with respect to their efferents to the cortex. A distinct localization to different cortical areas has been established mainly on the basis of Walker's (32) work with the method of retrograde degeneration after various cortical lesions.

Very little is known about efferents from the hypothalamus and subthalamus. So far, there is no reason to assume that efferents to the cortex arise in these regions since retrograde changes have not been reported after cortical lesions. However, both areas seem to send efferents to each other according to various studies of normal material (23). Evidence is also available that the hypothalamus, as well as the subthalamus, sends efferents to the thalamus. Relative data on this are unfortunately conflicting and one would wish some experimental data, if it were at all possible. One may assume with some certainty that the dorsomedial nucleus is connected with the hypothalamus, while there is not doubt of course about the efferents in the mammillothalamic tract (7).

Besides these ascending efferents, mention should be made of various efferents to the mesencephalon and more caudal brain stem regions. Some of these end in the reticular formation proper and others appear to end upon vegetative motor nuclei of cranial nerves with the bundle of Schütz. The exact origin of these descending efferents (and the same would apply to subthalamic efferents) needs further investigation. Retrograde degeneration is a difficult technique for these areas since the appropriate lesions have to be placed mostly in the reticular formation. Crosby (4) recently pointed out that such lesions are bound to lead to failure, or at least to conflicting results, since short neurons are mixed here with numerous unbundled long axons proceeding in the rostral, as well as caudal, direction.

These axons have been partly analyzed, particularly those derived from the basal ganglia by Papez (18). Lesions in such a complex area are apt to lead

nowhere when they are studied with the method of retrograde degeneration. It is doubtless true that long fibers ending upon such a network of short neurons are much more easily traced, which explains why evidence is now accumulating concerning afferent fibers to the thalamus, and particularly to the hypothalamus, subthalamus, and midbrain reticular formation. Lesions placed at a distance and destroying neurons directed to these areas constitute a more adequate method of investigation in this respect. The afferents of the diencephalon either end in specific relay nuclei, as is very well established by the Marchi method (e.g. Gerebtzoff and Wauters (6)) or in areas which belong to the most rostral regions of the reticular formation and which are characterized by the short neuronal patterns that I have described above. The afferents of the latter are not all clearly defined. Moreover, there is a considerable amount of overlapping.

When considering the thalamus, it is not sufficient to distinguish between principal nuclei on one hand, and median, intralaminar, and extralaminar nuclei on the other. It is true that the former show a topological distribution with respect to their afferents, but I have been able to find diffuse endings in them also after different lesions in the frontal lobe of the cat. This applies particularly to the nucleus ventralis posterior where degenerating terminals are found not only after lesions in the sensory cortex but also after ablations of the motor and premotor cortex. These studies have been carried out with a combination of two techniques, i.e. our own bouton method (2) and the Nauta method (16) for selective impregnation of degenerating fibers and terminals. The former has the disadvantages which I mentioned before but it is useful as a control for slides stained with the Nauta method. method impregnates, with few exceptions only degenerating fibers and their terminals, but it does not reveal the boutons. With the bouton method as control and also the normal side for comparison in material that has unilateral cortical ablations several afferent connections are now confirmed to the diencephalic reticular formation. I must point out that there is doubt concerning several contributions in this field that are based on the Glees (33) technique. The conflicting evidence obtained with this technique is understandable if I point out that I have seen sections of normal material stained with this modified Bielschowsky method in which several fibers showed beading, fragmentation, and other accepted signs of degeneration. Several afferents reported with this technique, e.g. by Glees, Wall, and Fulton (33), Meyer (13), and Le Gros-Clark and Meyer (12) need reinvestigation on this basis alone. I have not been able so far to find afferents, arising in cortex, to any of the hypothalamic nuclei although they have been frequently described in cat and monkey. The strychnine neuronography studies of Niemer and Castellanos (17) have hardly gone any further than reaching the conclusions that there are terminations of cortical origin in the hypothal-The authors were able to be more specific for the amus and subthalamus. thalamus.

The only reliable anatomical method to my mind is the combination mentioned above of a selective impregnation and a bouton method. The

Häggqvist method deserves to be tried but I have had no personal experience with it. It might be extremely difficult to follow scattered degenerated fibers in material stained with this method, particularly when the area is almost unmyelinated, as is the case in the hypothalamus. So far, frontal ablations in cat material have enabled us to reveal:

(1) Diffuse fibers from the gyrus proreus, gyrus sigmoideus anterior, and gyrus coronarius to the intralaminar nuclei, the nucleus reticularis, and the centrum medianum.

(2) Lesions in the gyrus proreus are followed by terminal degeneration in the dorsomedial nucleus to an extent proportional to the size of the ablation.

(3) Terminal degeneration in the zona incerta and subthalamic nucleus is always noticed after ablation of the frontal cortex. Degenerative fibers pass through the subthalamus towards the tegmentum.

(4) Terminal degeneration in the hypothalamus is restricted to the lateral hypothalamic area. Little evidence has been obtained in support of the existence of corticohypothalamic fibers in any of the principal hypothalamic nuclei as described by Meyer (13) and others.

So far the frontal lobe in the cat has been studied with this approach. However, observations are forthcoming concerning similar diffuse thalamic afferents from other cortical regions. The occipital area has been studied by Nauta (15) in the rat with results that fall in line with our own findings as regards the frontal lobe. It seems nevertheless that the frontal lobe is the main source of afferents to the head of the reticular nucleus. Other regions of the cortex are now being investigated in an attempt to find out whether diffuse connections to the diencephalon exist and whether any localization may ultimately be detected in these afferents.

Many problems besides this mapping remain unsolved. One of them is to find out how different elements of the principal thalamic nuclei are related to each other and to the unspecific nuclei. Circumscript lesions in the principal nuclei may lead to an understanding of their efferent connections with adjacent regions but I doubt whether similar experiments could be carried out in the diencephalic reticular formation. Anatomical methods presently available are insufficient to reveal anything about adjacent efferents of the unspecific nuclei for reasons that I have already pointed out with regard to other areas of the reticular formation.

The important relations between the mammillary bodies and the anterior thalamic nuclei (11) and similarly the now established endings of the fornix to the same anterior nuclei and to the hypothalamic reticular substance (8) must remain outside the scope of this presentation since their discussion would involve the vegetative nervous system and would oblige us to go into problems of hippocampal and limbic cortex as well. Sprague and Meyer (28) and Powell and Cowan (22) have quite correctly stated that particularly the former has acquired great significance in higher mammals, a significance far above that of the rhinencephalon in man. Clinical data (20) point to the significance of the temporal lobe, and its relations with the diencephalon

need to be mentioned in this respect. Similarly, the relations of the amygdaloid with the reticular formation are of great importance as has recently again been shown by Hunsperger (9), who found that rage reactions can be elicited by stimulation as far caudal as the midbrain reticular formation and as far rostral as the amygdaloid.

Finally, the question may be raised as to how the anatomical data discussed in this talk, and they are by no means complete, are related to the functional data published during the last 10 years. Neuronal corticothalamic circuits postulated by electrical recording techniques seem to have an anatomical substratum. This applies to circuits through the principal nuclei as well as through the diffuse nuclei. The latter however have certainly not more than a single neuron interposed in their thalamocortical complex (Powell and Cowan (22)). It is up to the physiologist, as well as the anatomist, to establish whether a multineuronal thalamocortical pathway is demonstrable which takes its origin from the intralaminar nuclei. The connections between the striatum and the diencephalon definitely need thorough study, the more so since recent experiments infer that the striatum (caudate nucleus) may also have an activating influence on the unspecific thalamic nuclei (26). These experiments postulate that connections should exist also to the nucleus ventralis anterior, from the caudate as well as from the cortex. I have personally not been able to find the latter, while the former are now being studied. Pallidal fibers to the nucleus ventralis anterior have been described by Ranson and his co-workers (16, 19). In conclusion, I should like to say that although much has been achieved in an attempt to unravel the complex corticodiencephalon relations, the principal problems as to how the different elements of the diencephalon influence each other and the cortex remain obscure. The assumption by Livingston and his co-workers that corticothalamic efferents, which I have described from the anatomical point of view, serve as a substratum for activation, sustaining, and control of awareness will doubtless become much clearer when we know more about the integrator of this function, i.e. the rostral reticular formation. The difficulty is increased by the fact that anatomists, as well as physiologists, are inclined and also expected to postulate their observations in terms of mental processes. Such a postulation presupposes bridges between functional events and psychological processes which have not been established.

Anatomists and physiologists both are aware of the need for further analysis of structural relations. So far, it may be concluded only that neuronal patterns in the hypothalamus, subthalamus, and a great part of the thalamus suggest that we deal with a rostral continuation of the midbrain reticular formation. It is also established that efferents from the cortex and from the striatum flow into this neuronal pool. Similarly, the long sensory pathways send collaterals into it besides their proper ending in the principal thalamic nuclei. Moreover, there is evidence also of cerebeller efferents terminating, via the brachium conjunctivum, in this pool (31). It stands to reason that this knowledge does not as yet permit more than the statement that these

structures may act as an integrator of activation. We have no idea how this task is performed under different conditions of inflow. It should even be considered as possible that individual variations in such a neuronal pool exclude the possibility of a detailed functional analysis and that we shall have to be satisfied with some sort of a common denominator from a functional point of view. A similar reservation has recently been made by Sholl (27) with respect to the structural pattern of the cortex.

The deluge of data coming to us from electrophysiological investigations suggesting connections which are either conflicting with each other or as yet undetectable by morphological methods should be a warning that we need to exercise caution in our interpretations of these results and seek improvements of methods, anatomical in any case. New methods are indeed urgently needed to unveil at least the general structural basis on which the rostral reticular formation acts as an activating integrator. Low power electromicroscopy as recently introduced by Young (34) might be of great help in order to analyze multineuronal pools encountered in this region. Perfection of staining techniques is another necessary task in order to find in normal upper brain stem material evidence of synapses, evidence which is scanty up to now. Though reservation is therefore necessary, the outlook seems to be encouraging in view of the possibilities, and it is no doubt on this basis that we have to continue our work in order to understand this complex and enchanting region of the brain.

References

- AUER, J. J. Comp. Neurol. 95: 17. 1951. Anat. Record, 115. 1953.
 AUER, J. and DI VIRGILIO, G. Stain Technol. 28: 141. 1953.
 BODIAN, D. J. Comp. Neurol. 72: 207. 1940.
 CROSBY, E. Proc. Neurobiology Congress, Groningen. 1955.
 DROGGLEEVER FORTUYN, J. Folia psych. neerl. 53: 213. 1950.
 GEREBTZOFF, M. A. and WAUTERS, A. Cellule, 48: 7. 1941.
 GUDDEN, B. VON Ges. u. hinterlassene Abhandl. Wiesbaden. 1889.
 GUILLERY, R. W. J. Anat. 89: 19. 1955.
 HUNSPERGER, R. Proc. Neurobiology Congress, Groningen. 1955.
 JASPER, H. H., AJMONE-MARSAN, C., and STOLL, J. Arch. Neurol. Psychiat. 67: 155. 1952.

- JASPER, H. H., AJMONE-MARSAN, C., and STOLL, J. Arch. Neurol. Psychiat. 67:155. 1952.
 LE GROS CLARK, W. E. Brain, 55:406. 1932.
 LE GROS CLARK, W. E. and MEVER, M. Brain, 70:304. 1947.
 MEYER, M. Brain, 72:265. 1949.
 MORRISON, R. S. and DEMPSEY, E. W. Am. J. Physiol. 135:281. 1942.
 NAUTA, W. J. H. Anat. Record Abstr. 1953.
 NAUTA, W. J. H. and GYGAX, P. A. Stain Technol. 29:91. 1954.
 NIEMER, W. T. and JIMENEZ-CASTELLANOS, J. J. Comp. Neurol. 93:101. 1950.
 PAPEZ, J. W. Research Publs., Assoc. Research Nervous Mental Disease, 26:21. 1940.
 PENFIELD, W. and JASPER, H.H. Epilepsy and the functional anatomy of the human brain. Little, Brown & Co., Boston. 1954.
 PENFIELD, W. and JASPER, H. Epilepsy and the functional anatomy of the human brain. Little, Brown & Co., Boston. 1955.
 POWELL, T. P. S. and COWAN, W. M. J. Anat. 88:307. 1954.
 POWELL, T. P. S. and COWAN, W. M. Brain, 78:115. 1955.
 RANSON, S. W. and MAGOUN, H. W. Ergeb. Physiol. biol. chem. u. exptl. Pharmakol. 41:56. 1939.
 ROMANES, G. J. J. Anat. 80:205. 1946; 84:104. 1950.
 ROMANES, G. J. J. Anat. 80:205. 1946; 84:104. 1950.
 SHIMAMOTO, T. and VERZEANO, M. J. Neurophysiol. 17:278. 1954.
 SHOLL, D. A. Proc. Neurobiology Congress, Groningen. 1955.

28. SPRAGUE, J. M. and MEYER, M. J. Anat. 84:354. 1950.
29. STARZL, T. E. and MAGOUN, H. W. J. Neurophysiol. 14:133. 1951.
30. VERHAART, W. J. C. Proc. Neurobiology Congress, Groningen. 1955.
31. VERHAART, W. J. C. Proc. VIth Intern. Congr. Anatomy, Paris. 223.

32. WALKER, A. E. The primate Thalamus, The University of Chicago Press, Chicago. 1938.

33. WALL, P. D., GLEES, P., and FULTON, J. F. Brain, 74:66. 1934. YOUNG, J. Z. Proc. Neurobiology Congress, Groningen. 1955.

Discussion: M. L. BARR¹

It is regrettable that Dr. Jasper is unable to attend this Symposium and discuss Dr. Auer's informative paper. My substitution for Dr. Jasper is a matter of expediency hope to open the discussion with the pertinency that he would have achieved My substitution for Dr. Jasper is a matter of expediency and I cannot

Neurophysiologists are no doubt curious concerning the reliability of anatomical methods that are used for tracing nerve fibers to their terminations. With this thought in mind, I will discuss methods very briefly and leave other important matters raised by Dr. Auer's

paper for comment by others.

The neurohistologist relies upon Wallerian degeneration very largely in tracing nerve fibers to their terminations after the proximal portions of the neurones have been damaged The Weigert and Marchi methods take advantage of degeneration of myelin experimentally. sheaths, and much of our knowledge of the course of fiber tracts in the central nervous system is derived from the use of these methods. They fall short of perfection in that degenerating unmyelinated fibers are not seen, nor are the terminal unmyelinated portions of myelinated fibers. Silver staining methods came to be used in order to fill this need, and the trend of events has been as follows.

I believe it was Sherrington who suggested that degenerating synaptic end-bulbs might be recognized with Cajal's reduced silver nitrate method. This was systematically investigated by Hoff and Hoff, for corticospinal endings in the first instance, who showed that it is possible to recognize degenerating synaptic endings since they are swollen, have an increased affinity for silver, and are fragmented at some stages of degeneration. The bouton degeneration for silver, and are fragmented at some stages of degeneration. The bouton degeneration method has produced some valuable information. However, the histologist is well aware of sources of error in the method. They arise principally from the vagaries of empirical silver methods and from the fact that there is a good deal of variation, in size and other details,

among normal synaptic end-bulbs.

Because of this, a search was made for methods of staining degenerating axis cylinders selectively, so that these methods could be used in conjunction with the bouton technique or as an alternative to it. Various modifications of the silver method were developed for this purpose by Nauta, Glees, and others. The feature held in common by these stains is that fibers degenerating as a result of surgical damage to the proximal parts of the neurones can be recognized because of their increased affinity for silver, irregular swelling (beading), and fragmentation. These methods are applicable to the terminal branches of nerve fibers in the vicinity of their synaptic contact with other neurones, as well as to the main axis cylinders. As in the bouton degeneration method, meticulous care is required in preparing the sections and in their interpretation.

The reliability of the silver staining methods for studying connections between various areas of gray matter may be assessed as follows. The methods are capable of yielding the information sought for, but they are by no means perfected to the point where they can be applied casually. It is necessary to learn the capabilities and weaknesses of the methods by personal experience, as Dr. Auer has done. Improvements are certain to occur, since the various steps in the silver procedure are being investigated chemically in order to reduce their empirical nature, and since non-metallic staining methods are being introduced for the purpose of tracing degenerating axis cylinders. Methods are also being sought for establishing purpose of tracing degenerating axis cylinders. Methods are also being sought for establishing the connections of the important Golgi type II neurones, since the present methods are

unsuitable for this.

When using the silver methods personally, I have the feeling that the information sought can be made available, but that the greatest care must be exercised constantly to avoid errors that can arise from faulty staining technique or uncritical interpretation of the sections. I am sure that this is the attitude of others who, like Dr. Auer, use the silver methods a good deal, and who have experienced frustrations as well as successes. This attitude will strike a responsive note among neurophysiologists who use the even more complicated electronic methods to search out neuronal connections. Progress in working out the finer connections of the brain requires, in fact, simultaneous contributions from those who prefer to examine the connections visually, and those who search for them by inference from the results of electrical recordings and other devices.

¹Contribution from the Department of Microscopic Anatomy, University of Western Ontario, London, Ontario.

THE ELECTROPHYSIOLOGICAL APPROACH TO THE PROBLEM OF LEARNING¹

By B. DELISLE BURNS

If a friend were to ask you why running upstairs made him out of breath, or how 2 pints of beer increased his urinary output, or by what magic an ingested biscuit becomes capable of contributing to a symposium soon after it is eaten—you would be able to answer in some detail. Many parts of your answers to such questions could be given in quantitative terms and provided your friend knew a little physics and chemistry it would be easy to send him away intellectually satisfied. But if this persistent and annoying character were to question you concerning the physiology of memory—say he were to ask "How do I remember my way home from here?", there would be little you could say to rescue your prestige as a physiologist.

The physiology of learning virtually does not exist. As a subject it still belongs more to the philosopher than the scientist, for its definitions and hypotheses can multiply with little fear of obstruction from measurement or experimental data. Nevertheless, the lack of knowledge in this field is certainly not due to lack of scientific interest or effort. Many physiological mechanisms for learning have been suggested, and some have been tested, but so far none has been found wholly satisfactory.

When something is learned by an animal its reactions to a certain stimulus or combination of stimuli become changed for a long period of time. Afferent excitation of the central nervous system, which before learning produced a complex of efferent activity 'A', or perhaps, no detectable activity, after learning produces new efferent activity 'B'. It is difficult to avoid the conclusion that the paths most readily available to the relevant nerve impulses within the central nervous system have been altered during the learning process. The activity set up by the incoming afferent stimulation has been rerouted. conclusion does not imply, of course, that the central nervous system should be regarded as a complicated mechanical switchbox within which each learned response is represented by one fixed pathway of low resistance. Lashley (19, 20) pointed out a long time ago that no learned reaction involving skeletal muscles is ever completed twice by precisely the same series of muscular movements. It is the end result which is constant. Nevertheless, statistically speaking, the most probable pathways available to afferent nerve impulses within the central nervous system must have changed after something is learned, and it is the nature and site of this presumed rerouting of activity which has persistently interested neurophysiologists. Put in this way, the

¹Manuscript received December 13, 1955. Contribution from the Department of Physiology, McGill University, Montreal, Que. This paper was presented at the Symposium on Neurophysiology held as part of the Annual Meeting of the Canadian Physiological Society, London, Ontario, October 13–15, 1955. neurophysiologist can avoid the necessity of attempting too precise a definition of learning. For the moment he would be happy to find any nerve cells or set of synaptic junctions whose properties of conduction he could change at will, for long periods of time.

Because neurophysiological concepts are simple, they are frequently disguised with very long names. Therefore, before trying to summarize some of the neurological mechanisms that have been considered as possible components in the mechanism of learning, I think it would be kind to those who are unaccustomed to electrophysiological jargon to outline the few fundamental properties of nerve cells upon which my arguments will depend.

At rest, a nerve cell, which consists of cell body and processes, shows a potential difference between the outer and inner faces of all parts of the limiting cell membrane. The outside of the cell membrane is positive to the inner face—it is electrically polarized; this resting membrane potential can be measured by having one electrode of a voltmeter in electrical contact with the outer face of the cell and stabbing the other electrode, which of course must be very small, through the cell membrane and into the interior of the cell. In this way a potential of 50-100 mv. is recorded (Huxley and Stampfli (16), Eccles (9)). Excitation of such a cell is always started by forces which bring about a local reduction in the resting membrane potential at some point on the cell membrane. It is found that any process which reduces the local membrane potential (or depolarizes) suddenly by more than 15% causes the origin of a spreading action potential during the passage of which the cell membrane ceases to be a good insulator and allows temporarily the complete collapse of the resting charge (Hodgkin, Huxley, and Katz (15), Fatt and Katz (13), Brock, Coombs, and Eccles (2)). The result is a wave of activity which invades all parts of the cell. After the action potential has passed, usually within a few milliseconds, resting membrane potential is restored by some energy-consuming process. The conducting properties of the long processes or axons of nerve cells are surprisingly stable. The velocity with which a single action potential traverses an axon in either the tracts of the central nervous system or in peripheral nerve is almost the same as that of a continuous stream of action potentials at 100/sec. The excitability and other functional properties of axons seem almost as resistant to fatigue, and to changes in their external environment as the copper wire of a telephone system. They do not show the flexibility of function which must be present in a system that can learn. The transmission of activity only becomes hazardous or variable when it must pass from one nerve cell to another. In such a simple system of two cells and their points of functional contact, the mechanisms that determine the nature of transmission lie in the region which includes the terminal endings of the first or afferent neurone, the minute points of synaptic contact, and the cell body and short processes of the efferent neurone. It is the properties of these structures which determine the ease with which the first neurone may excite the second. Thus where two electrically conducting cells make functional contact one of or a number of things may happen:

- (1) A single action potential in the first cell may not be capable of exciting the second cell. All it can do is to produce a local subthreshold depolarization at the points of synaptic contact. Nevertheless by so doing it will have increased the excitability of the efferent cell so that a second afferent action potential arriving hard on the heels of the first may succed in exciting the efferent cell.
- (2) A single action potential in the first or afferent cell may invariably cause an action potential in the second cell.
- (3) A single action potential in the first cell may cause a whole train of action potentials to be discharged by the second or efferent cell.

As you can see we have made a list of three classes of decreasing junctional resistance. In the peripheral nervous system and within the spinal cord, junctions of all three types have been found. There are synapses within autonomic ganglia which in the rested state will not allow the passage of a single afferent action potential, but will allow the passage of the second of a pair of incoming action potentials (Bronk (3)). Some junctions of motor nerve with skeletal muscle in the chicken behave in the same way (Brown and Harvey (4)); while a single action potential in the motor nerve will not cause a twitch from the muscle fiber, two action potentials succeed. Unlike those of the chicken, mammalian neuromuscular junctions transmit every action potential in the rested state and therefore belong to the second class of synapse that we have listed. Only when treated with curare do they behave like those in the fowl (Eccles, Katz, and Kuffler (11)). Finally, there are cells within the spinal cord called Renshaw interneurones which respond to a single afferent stimulus by emitting a whole train of action potentials at very high frequency (Eccles, Fatt, and Koketsu (10)). More important to our present interest is the study of junctional regions whose resistance to the passage of action potentials varies with variation in the amount of their recent activity. We have already mentioned that some junctions between pre- and postganglionic autonomic nerves are incapable of passing a single action potential in the rested state. Nevertheless, after a period of bombardment with afferent impulses these junctions become able to transmit every incident action potential (Larrabee and Bronk (17)). Moreover, provided the afferent bombardment from the preganglionics is very severe, the postganglionics will continue to discharge action potentials for many seconds after stimulation has ceased (Larrabee and Bronk (18)). It appears then that the resistance which an autonomic ganglion presents to transmission can be decreased by previous or conditioning activity in the preganglionics; in fact the junctional resistance can be reduced to a negative quantity in the sense that ultimately an afterdischarge takes place and for a limited time the postganglionic cells fire spontaneously.

The facilitations that I have mentioned have each provided useful information concerning potential mechanisms by which junctional resistance may vary physiologically. Nevertheless, these examples are disappointing in that none of the facilitations we have discussed persists for more than a number of seconds. Facilitation in the partly curarized neuromuscular junction lasts fractions of a second. The facilitation of the stellate ganglion demonstrated by Larrabee and Bronk lasted at most a minute. A longer lasting and therefore more encouraging form of facilitation has been studied by Eccles in the spinal cord (Eccles and McIntyre (12)). Despite the anatomical complexity of neurone connections within the cord methods have been worked out by which the synapses which join sensory fibers from muscle spindles to motoneurones can be functionally isolated from the rest of segmental activity. In this particular reflex are, only one synapse lies between the incoming sensory fiber and the outgoing motor-nerve cell (Lloyd (21)). When precautions were taken to prevent any afferent stimulation reaching these junctions for a number of weeks, it was found that the synapses developed great resistance to the passage of excitation. Nevertheless, these resistant junctions could have their normal conductivity restored provided the afferent nerves were repeatedly stimulated at high frequency for a period of a few seconds. The facilitating effect of this tetanic stimulation remained measurable for a number of hours afterwards.

Such experiments as these have made it clear that, where there is a junction between two nerve cells, facilitation of transmission can occur as a result of at least three mechanisms:

(i) The algebraic summation of local subthreshold depolarizations of the efferent cell membrane. These local depolarizations may add until finally they are sufficient to cause a spreading action potential, simply because the efferent cell membrane takes a short measurable time to repolarize. (The facilitations of fowl muscle or of part curarized mammalian skeletal muscle would fall in this category.)

(ii) The slow destruction or dispersal of a chemical agent or humoral transmitter liberated by the afferent cell terminals. Specialized, sensitive sites on the efferent cell are maintained in a partially depolarized condition by the persistence of the transmitter with the consequence that the excitability of the efferent cell is raised. (Examples of this mechanism would be facilitations of Renshaw's interneurones and the

early stages of facilitation in autonomic ganglia.)

(iii) Modification in the shape of the afferent nerve terminals in such a way that they make more effective contact with the efferent cell membrane. It has been shown that activity causes measurable swelling in peripheral nerve fibers and a similar swelling is supposed to affect the afferent nerve terminals after a period of great activity (Hill (14)). This hypothesis was used by Eccles and McIntyre to explain the long lasting facilitation that they were able to produce in artificially rested synapses.

Neither the spinal cord nor the peripheral nervous system can learn in any unmodified sense of the word (Sperry (24)). Such changes as can be inflicted on the properties of their nerve cells are relatively short-lived. Nevertheless, the detailed studies of these changes has provided ideas about facilitatory mechanisms, which with minor modification, may prove applicable to the case of longer lasting changes in the upper central nervous system.

Since all the evidence is that mammalian learning is conducted north of the neck it would seem sensible to search for the relevant mechanisms in the brain. For this reason considerable interest attaches to facilitatory mechanisms operating in the cerebral cortex. The cerebral cortex has been the center of neurophysiological attention in this respect for two reasons: first, its phylogenetic development appears to parallel the development of learning ability; second, it is very accessible. The interpretation of experiments with this organ is made particularly difficult, however, by the fact that its nerve cells are normally continually active, and because little is known of its microanatomical structure. Because of this lack of anatomical knowledge the results of many experiments must be temporarily interpreted on the assumption that the cortex is a homogeneous mass of neurones with their interconnecting synaptic junctions. least it is known that most neurones of the cortical gray matter have processes which spread only a few millimeters (Scholl (23)), so that activity which spreads tangentially throughout the gray matter must be transmitted from neurone to neurone by way of synaptic contacts. It was shown by Adrian (1) that repeated artificial excitation of a group of cortical neurones produces a decrease in the resistance of the synapses which surround these neurones so that with repeated stimulation the responses spread further and further across the cortex with each new stimulus. Such experiments show that some junctions between cortical neurones behave in a manner which is similar to that already observed in autonomic ganglia. Moreover, as with autonomic ganglia the continuation of a stimulus which leads to facilitation can cause a prolonged afterdischarge when stimulation ceases. This has been termed a paroxysmal or epileptiform afterdischarge because the general form of electrical activity looks so like that seen in cases of clinical focal epilepsy (Penfield and Jasper (22)). Unfortunately, however, the cortical synapses as studied by Adrian's method appeared too like those of autonomic ganglia; neither the facilitations produced by artificial conditioning stimulation nor the afterdischarge last very long. Prof. Adrian (1) said "the pathways formed by summation from one neurone to another are of the simplest character and have a life reckoned in seconds or at most minutes after stimulation has ceased. There is no evidence that they can ever become complex enough or permanent enough to be the basis of a learnt reaction".

The brief survey I have given you of the immense effort which has been put by neurophysiologists into the investigation of facilitatory mechanisms is no more than a history of failure where the physiology of memory is concerned. We have arrived at the point where in most symposia the contributor modestly describes the way in which he solved a previously invincible problem. But I am afraid I must disappoint you. All I can do is to spend the remaining few minutes describing a series of experiments which uncovered one more mechanism for facilitation—a mechanism which, like the others I have discussed, proved a disappointment.

This series of experiments have all been carried out with the cat's neurologically isolated cerebral cortex (Burns (5)). In the preparation for such an experiment a cut is first made so as to separate the brain from the spinal cord and then the animal is taken off ether. An area of cerebral cortex of about one square centimeter is then undercut in such a way that all its connections with the rest of the nervous system are severed, without damage to its blood supply, which fortunately runs in from the pial covering on the surface. There were two reasons for using such a preparation:

- The presence of even very little anesthetic was known to interfere with the normal processes of neuronal transmission.
- (2) The isolation of an area of cortex simplifies the activity within it and in fact gives the experimenter full control over its behavior.

When a group of cortical neurones is isolated in this way the continuous, so-called spontaneous activity of intact brain dies down and most if not all of the isolated neurones become inactive unless excited artificially. There is some argument about how inactive isolated neurones become: but all those who have tried isolating cortex are agreed that the operation produces a great reduction of net activity within the gray matter (Burns (7)). In our hands, isolated cortical neurones become quite silent and although this may be an unphysiological consequence of surgical technique it is a particularly convenient one. Whatever damage may have been done to the assembly of isolated nerve cells many of them will readily respond to stimulation. In fact perhaps the most surprising feature of isolated cerebral cortex is the constancy and predictability of its behavior. All research from that of Pavlov to that of Penfield has pointed to the cerebral cortex as an organ intimately concerned with elementary memory processes. Yet a weak artificial electrical stimulus given to the surface of isolated cortex gives a response the characteristics of which are as predictable as those of a skeletal muscle when its motor nerve is excited. When I first isolated a piece of cortex alive I must admit that I expected to find a preparation that was wayward and fickle in its behavior for I romantically imagined that I had dissected free a small part of the cat's past history! Perhaps the explanation of the machine-like behavior I have described is that current methods of excitation and of recording are too crude, for beneath each square millimeter of pial surface lie 40,000 neurones so that an artificial stimulus from a wire electrode must excite simultaneously many cells which normally should never fire together. Nevertheless, when a response is elicited by such highly artificial means and spreads to neurones far beyond the reach of the stimulating current, we can be sure that its spread is by normal physiological routes. Thus, a relatively weak stimulus of one millisecond duration given to one end of a slab of isolated cortex always elicits a response which spreads like a wave across a water surface to invade the whole isolated area. The response spreads indefinitely in all directions and without attenuation at about 15 cm./sec. It is apparently handed from neurone to neurone within a network of cells lying about a millimeter below the brain's surface. One of the curiosities of this response to a single stimulus is that each neurone, once it has been excited by the spreading wave front, discharges many times so that the response lasts several seconds at any given cortical point. No one has yet identified under the microscope the neurones which give this burst response, but physiological tests have given us a fairly good idea of their site and shape (Burns and Grafstein (8)). We believe them to be spread throughout the gray matter as a system of functionally interconnected cells with their somata about one millimeter down, and with synaptic connections a little below this depth. Since no histologist has yet identified them we have temporarily christened them type-B cells. Although their contribution to the behavior of the intact animal is not known, this network of cells seems to spread over most of the cat's cortex; moreover a network of cells giving very similar responses appears to exist in a number of other animals which includes the monkey and man. The type-B network is particularly interesting for two reasons. First, it is apparently capable of conducting excitation from any cortical point to any other point by a route entirely within the gray matter. Second, it holds the world's record for afterdischarges. Ten single stimuli, each of one millisecond duration, can cause the stimulated point to become an origin for spontaneous bursts of activity, which continue for one-half to one hour after stimulation has ceased (Burns (6)). Each burst in the series looks exactly like the response of rested cortex to a single stimulus, so that during such a series of afterbursts one might easily believe that the stimulator had not been shut off.

In a hunt for memory mechanisms, a preparation in which 5 or 10 stimuli could produce a dramatic change in properties for half an hour or more was very encouraging: and an investigation of the properties of this case of facilitation soon led to an hypothesis concerning the underlying mechanism, which although not proved, is simple and still fits all of the available facts. To make things a little clearer I will reverse history and give you the hypothesis first and afterwards outline some of the facts that gave rise to it. When type-B neurones beneath the stimulating electrode are excited, as with other nerve cells a wave of depolarization sweeps over the whole of each neurone, discharging the membrane potential completely. But unlike other neurones, it is supposed that repolarization towards resting membrane potential proceeds more slowly for the deep or somatic end of the cell than it does for the process which runs towards the brain's surface. Thus at a time when the upper part of the cell is fully repolarized the lower part of the cell is still only partially In such circumstances current must flow in the interstitial fluid from the fully recovered membrane towards the less charged membrane. Now it is known from studies on other nerve cells that when two neighboring segments of membrane are unequally polarized, if the current flow between them exceeds a critical value then an action potential will originate and depolarization will invade all parts of the cell. Thus the picture we have of the production by a few stimuli to type-B cells of a long series of afterbursts is as follows. The period of conditioning stimulation fatigues the cells beneath the stimulating electrodes and during recovery from the driving stimulus one part of these cells recovers more slowly than the rest. A steadily increasing current begins to flow from one part of the cell to another until a spontaneous action potential occurs—this is the origin of the first afterburst in the series. But also this spontaneous burst sets the recovery process back one step and the whole cycle of events recurs, causing the second afterburst, and so on. This picture of a series of events, which have been termed differential repolarization (Burns (7)), implies that each burst of activity is the immediate cause of the next burst of activity in that each discharge of the type-B cells renews the conditions necessary for their spontaneous discharge. In support of this hypothesis are the following facts:

- (1) Any procedure which prevents one of a series of afterbursts stops the whole series. Thus if the blood supply to the cortex is clamped for 30 sec. during a series of afterbursts which one would expect to continue for say 10 min. the afterbursts stop permanently, although after restoration of circulation the cortex rapidly regains its original excitability.
- (2) Chronic artificial depolarization of the lower ends of type-B cells such as can be produced by forcing a small continuous current inward through the cortex makes these neurones discharge with a frequency rectilinearly related to the current flow—in fact a series of bursts originate from the region of forced depolarization.
- (3) The converse experiment is one in which a small outward current flow is forced through the cortex and is calculated to hasten repolarization of the somatic ends of the type-B cells. Switching on such a current will immediately halt a series of afterbursts.
- (4) During a series of afterbursts a small potential gradient can be recorded between an electrode 1 mm. deep and the surface above, indicative of the slow repolarization of the deep ends of the type-B cells. Moreover, the frequency of afterbursts is clearly related to this potential gradient.

These and other facts justify the addition of differential repolarization to the list of mechanisms that have been offered in explanation of various facilitations. Unfortunately, however, encouraging as the behavior of type-B cells looked three years ago, differential repolarization can have little to do with memory. True, they provide an unstable system, which like the pendulum of a grandfather clock, if caused to swing once, will remain active for a long period afterwards. But just like the pendulum, if one swing is prevented, all activity ceases. Such contribution to long-lasting changes within the central nervous system as may be made by the differential repolarization of type-B cells is distressingly easily wiped out. As Adrian said at the end of a similar study, there is no evidence that it "can ever become complex enough or permanent enough to be the basis of a learnt reaction".

So you see, the study of facilitations in the peripheral nervous system, in the spinal cord, and within the brain itself has turned up many mechanisms, each of which may well have to be fitted into our final picture of the changes produced by learning, but none of which alone suffices to make these changes intelligible. I believe that time may show that the presumed growth or movement of synaptic contacts described by Eccles and McIntyre is more closely related to learning mechanisms than any other process we have discussed here.

But despite the enormous accumulation of knowledge of the ways in which one neurone can effect another we can still only guess the answer. One day, I suppose, someone will find the clue and we shall then realize that we have been watching the missing mechanism at work in every experiment upon the brain that we did, but never recognized it for what it was.

References

1. Adrian, E. D. J. Physiol. 88: 127. 1936.
2. Brock, L. G., Coombs, J. S., and Eccles, J. C. J. Physiol. 117: 431. 1952.
3. Bronk, D. W. J. Neurophysiol. 2: 380. 1939.
4. Brown, G. L. and Harvey, A. M. J. Physiol. 93: 285. 1938.
5. Burns, B. D. J. Physiol. 112: 156. 1951.
6. Burns, B. D. J. Physiol. 125: 427. 1954.
7. Burns, B. D. J. Physiol. 127: 168. 1955.
8. Burns, B. D. J. Physiol. 127: 168. 1955.
9. Eccles, J. C. The neurophysiological basis of mind. Clarendon Press, Oxford. 1953.
10. Eccles, J. C., Fatt, P., and Koketsu, K. J. Physiol. 126: 524. 1954.
11. Eccles, J. C., Katz, B., and Kuffler, S. W. J. Neurophysiol. 4: 402. 1941.
12. Eccles, J. C. and McIntyre, A. K. Nature, 167: 466. 1951.
13. Fatt, P. and Katz, B. J. Physiol. 115: 320. 1951.
14. Hill, D. K. J. Physiol. 111: 304. 1950.
15. Hodgkin, A. L., Huxley, A. F., and Katz, B. J. Physiol. 116: 424. 1952.
16. Huxley, A. F. and Stampfli, R. J. Physiol. 112: 476. 1951.
17. Larrabee, M. G. and Bronk, D. W. Am. J. Physiol. 123: 126. 1938.
18. Larrabee, M. G. and Bronk, D. W. Proc. Soc. Exptl. Biol. Med. 38: 921. 1938.
19. Lashley, K. S. Physiol. Proc. 27. 44: 460.
20. Lashley, K. S. Physiol. Proc. 27. 44: 460.
21. Lashley, K. S. Physiol. Proc. 27. 44: 460.
22. Lashley, K. S. Physiol. Proc. 27. 44: 460.
23. Lashley, K. S. Physiol. Proc. 27. 44: 460.
24. Lashley, K. S. Physiol. Proc. 27. 44: 460.
25. Lashley, K. S. Physiol. Proc. 27. 44: 460.
26. Lashley, K. S. Physiol. Proc. 27. 44: 460.
27. Lashley, K. S. Physiol. Proc. 27. 44: 460.
27. Lashley, K. S. Physiol. Proc. 27. 44: 460.
28. Lashley, K. S. Physiol. Proc. 27. 44: 460.
29. Lashley, K. S. Physiol. Proc. 27. 44: 460.
20. Lashley, K. S. Physiol. Proc. 27. 44: 460. Foundations of experimental psychology. Clark Univ. Press, Worcester,

LASHLEY, K. S. Foundations of experimental psychology.
 Mass, 1929.
 LASHLEY, K. S. Physchol. Rev. 37: 1. 1930.
 LLOYD, D. P. C. J. Neurophysiol. 6: 316. 1943.
 PENFIELD, W. and JASPER, H. Epilepsy and functional anatomy of the human brain.
 Little, Brown & Co., Boston. 1954.
 SCHOLL, D. A. J. Anat. 89: 33. 1955.
 SPERRY, R. W. Quart. Rev. Biol. 20: 311. 1945.

DISCUSSION: J. W. PEARCE¹

It is an axiom, accepted by many physiologists who hold a mechanistic view of mental function, that the process of learning is initiated by activity of the sensory apparatus. Nervous receptors translate environmental changes into experience, and this experience may result in responses which, rendered appropriate by learning, succeed in affecting beneficially the equilibrium of the animal. Knowledge of receptor function has become extensive through the application of various techniques for recording the electrical activity in single sensory nerve fibers. Information is accumulating which shows how the sensory nerve signals are altered by conduction over synapses and by interaction with impulses from interconnected receptors. Much is still unknown, however, of the mechanism of these alterations in signal character and of their significance in central nervous system activity. This brief discussion will be restricted to a few examples of receptor activity which illustrate general principles, to a mention of some difficulties in interpretation of potentials recorded from central sensory areas, and to a suggestion of theoretical approach which might merit discussion.

There is a growing list of receptors of which the activity has been studied by single fiber recording techniques. For many receptors, where the exact nature of the physical stimulus can be measured and controlled, an intimate relation between stimulus and response has been shown to exist. This is illustrated by records of discharge in the single aortic pressoreceptor fiber and in a single auditory nerve fiber (4). The stimulus is reproduced qualitatively and quantitatively in the case of the first receptor and in each example the frequency of discharge bears a direct relationship to the stimulus intensity. Many receptors show adaptation, at a characteristic rate, to a continued stimulus of fixed intensity. Most visceral and proprioceptive receptors adapt only slowly, while cutaneous receptors, other than those of pain fibers, usually adapt rapidly. It is interesting that this adjustment, which usually exists where the stimulus does not require a persistent homeostatic response by the animal, occurs in the peripheral mechanism. the peripheral mechanism. Receptors are normally only excited by a specific stimulus. In

¹Contribution from the Department of Physiology, University of Alberta, Edmonton, Alberta.

the case of visceral and auditory receptors, the individual unit responds to a range of wave length of light or sound, specificity being achieved by maximum activity corresponding to a point or narrow width of the range (4, 5). At least one receptor has been shown to include a means for regulation of its own mechanical response to stimulation. This is the proprioceptive muscle spindle which is provided with intrinsic muscle fibers innervated by γ -sized myelinated efferents. The mechanism has been extensively studied by Hunt and Kuffler (6) who have shown that stimulation of the \gamma-efferents will result in discharge from the sensory apparatus of the spindle. This permits continued discharge even when the spindle is shortened by active muscle contraction. Katz has provided evidence (7) that muscle spindles are depolarized by deformation, resulting in development of a local potential which leads to a propagated impulse the connected nerve. A similar mechanism is probably common to most receptor endings. As a general principle, then, active receptors initiate nerve impulses of constant size and in the connected nerve.

duration, the only variation in signal content being change in discharge frequency and in number of active units. Central nervous system appreciation of the type of stimulus must depend on anatomical connections, and variations in the nature of the specific stimulus must be indicated by changes in frequency pattern of the impulses delivered to the sensory nuclei.

There is much evidence that the burst of impulses recorded from a receptor nerve may be altered by transmission across synapses. Microelectrode recording from sensory nerve nuclei at various levels may reveal discrete action potentials (2), or the impulses may be changed into or accompanied by slow potential changes (1, 3). The latter type of change is seen, for example, in records of evoked potentials from the somesthetic association area. The shape of the slow potential change may carry information of discharge frequency pattern. Interpretation of such potentials is complicated, however, by variations in shape depending on the position of the microelectrode point relative to the central neuron and on the electric field effect which may vary with the direction of approach of the potential change. Such variations are illustrated by the work of Tasaki, Polley, and Orrego (10). Even where the sensory impulses do retain their single unit character, interaction at synapses with other sensory axons may greatly alter the relationship between stimulus and response. Functional integration may may greatly alter the relationship between stimulus and response. Functional integration may have begun, with inhibition or facilitation. Kuffler's recordings (8) from single ganglion cells of the retina (third order neurons) illustrate inhibition resulting from interaction of sensory receptors in different receptive areas. This stresses the necessity for stage by stage recording of the transformations in sensory nerve signals. Certain visceral receptors discharge rhythmically and variations in frequency pattern can be readily induced by altering the function giving rise to the natural stimulus. Examples of such receptors are carotid pressoreceptors, lung stretch receptors, and atrial stretch receptors (9). It is probable that the pathways of such receptors would be simpler than those of somatic sensory mechanisms, and systematic recording along these routes might prove especially fruitful.

Reverting to the topic of mechanisms of learning, this process usually includes readiness of advantageous reaction to various stimuli. Some cyberneticists believe that it is profitable to consider reflexes as results of 'negative feedback' activity. Most simple homeostatic mechanisms are provided with a receptor; variation in activity of the receptor results in a response designed to oppose this change in receptor activity by correction of the stimulating environmental change. Learned responses can also be considered to be dependent on negative feedback from stimulation, internal or external, which tends to upset the physical or mental equilibrium of the animal. A mistaken response would result in aggravation of the still that might in some way inhibit the pathways active at that moment. Such inhibition could have persistent effects. The correct pathways would thus be facilitated by virtue of the absence of persistent effects. inhibition. Inhibition would then be the dominant process in learning, rather than that of active facilitation. As a point for discussion, is it possible that lasting changes in function or structure of nerve cells could be more readily produced by inhibitory influences than by

excitatory ones?

1. AMASSIAN, V. E. J. Neurophysiol. 17: 39, 1954.
2. COOPER, S., DANIEL, P. M., and WHITTERIDGE, D. J. Physiol. 120: 514. 1953.
3. GALAMBOS, R. J. Neurophysiol. 15: 381. 1952.
4. GALAMBOS, R. S. Neurophysiol. 6: 39, 1943.
5. GEANIT, R. J. Neurophysiol. 6: 195. 1945.
6. HUNT, C. C. and KUFFLER, S. W. J. Physiol. 113: 298, 1951.
7. KATZ, B. J. Physiol. 111: 261. 1950.
8. KUFFLER, S. W. J. Neurophysiol. 16: 37, 1953.
9. PEARCE, J. W. and HENRY, J. P. Federation Proc. 13: 109. 1954.
10. TASAKI, I., POLLEY, E. H., and ORREGO, F. J. Neurophysiol. 17: 454. 1954.

œ.

PHARMACOLOGICAL AND ENDOCRINOLOGICAL INFLUENCES ON MENTATION AND EMOTIONS¹

By R. A. CLEGHORN

The history of the use of drugs affecting mood goes back into the dim antiquity of mankind, and includes as prominent members, alcohol and the poppy seed (81). Some, such as coffee, have been introduced and used at such a social level that we barely stigmatize them with the label 'drug' (12). Not many of the drugs now so widely used, including the barbiturates, produce psychotic changes, but amphetamine may. Drastic consequences are the exception with this adrenalin-like compound, though psychoses do occur when it is taken in excess (42).

The infrequency of severe mental disturbances with the barbiturates or benzedrine derivatives, and the inadequacy of definitive explanation for their action on the brain, meant that they had a limited impact on psychiatric theory, despite the interest in the abreactive effects. The introduction of drugs which regularly produced reversible psychotic-like states, within recent years excited widespread interest in scientific and lay circles. Gradually it became apparent that they might be used as keys to unlock some of the secrets of psychopathology.

Of these drugs, the two chief are mescaline and d-lysergic acid. They might have remained clinical curiosities if the climate of opinion had not been right for their utilization for systematic inquiry, and if this had not been followed by clues to an understanding of the chemical processes involved. It is of signal interest to us today that these two compounds bear a certain and possibly significant resemblance to epinephrine, as does amphetamine.

Concurrent and almost wholly unanticipated developments in the field of endocrinology now appear to be not simply fortuitous, but an integral part in the opening of the doors of psychopathology implemented by the pharmacological approach. These findings, namely, the striking changes in mood and the occurrence of psychotic states in patients receiving cortisone and ACTH, were first reported about five years ago. It is with these two areas of advancing knowledge, the pharmacological and endocrinological, and the means they give us for attacking and understanding pathological developments in thought, mood, and perception, that I wish to deal today.

Before proceeding to a detailed consideration of those drugs and hormones which produce deviation in affective and thought processes, I would like to draw the attention of those interested in interdisciplinary things to that felicitous article by Ralph Gerard (35) on the "Biological roots of psychiatry".

¹Manuscript received December 13, 1955.

Contribution from the Allan Memorial Institute of Psychiatry and Department of Psychiatry, McGill University, Montreal, Que. This paper was presented at the Symposium on Neurophysiology held as part of the Annual Meeting of the Canadian Physiological Society, London, Onlario, October 13-15, 1955.

My remarks may complement, though in no wise imitate, his. In order to establish a base line for subsequent considerations, it is necessary, first, to review certain relevant data concerning the adrenal and autonomic nervous system.

A. The Adrenals and the Autonomic Nervous System

(1) Putative Relationships Between the Adrenal Cortex and Medulla

The adrenal is a compound gland derived embryologically from two very different sources. The cortex arises from the coelomic epithelium, near the root of the mesentery, adjacent to the genital ridge, which is the origin of the gonads. It is little wonder, therefore, that they later secrete many similar hormones, steroids. The medulla has its origin in common with the neuroblastic ectodermal mass which later develops into the sympathetic ganglia. The reason for the increasingly close association of these two disparate tissues as one ascends the phylogenetic scale, or in ontogeny, is almost wholly mysterious. The intimacy of function of these separately originating parts of what is apparently a functioning unit is further attested to by the disposition of the blood supply. This flows from the cortex into the medulla. Functionally the two parts seem to be related in that the medulla secretes at once in response to various stresses to the organism; the cortex responds to most similar stresses, especially if they are maintained, in a slower and more enduring way (56). The medullary secretion appears to be part of the emergency autonomic defense, the cortical secretion a hormonally mediated attempt on the part of the body to master and survive more sustained stress. And in this process epinephrine is probably involved in releasing ACTH to stimulate the cortex.

The writer was preoccupied for some years with the apparent failure of the sympathetic nervous system to respond adequately to stimulation after adrenalectomy and the cardiovascular failure in adrenal insufficiency, because this approach seemed to provide a possible link between adrenal cortex and medulla (4, 16). This is not the place to itemize those findings, but reference must be made to the significant experiments of Levine and co-workers (31) who pursued the matter further. These investigators found that the atonic vessels of the adrenalectomized rat became refractory to norepinephrine. Responsiveness was restored by administration of adrenocortical extract (ACE), but not by desoxycorticosterone. This indicates that 11-oxygenated glucocorticoids are necessary to permit norepinephrine to function. There is also evidence that this is true for epinephrine (21).

There is still another avenue of research which has demonstrated a dependence of the adrenal medulla upon the cortex. This was inspired by von Euler's intensive investigations of norepinephrine. In this systematic study of conditions affecting the norepinephrine and epinephrine content of mammalian tissues Hokfelt (48) found that hypophysectomy led to an increase in norepinephrine and a decrease in epinephrine in the adrenal medulla. An injection of ACTH, thereby stimulating the cortex, had the opposite effect.

The implication of these findings is not yet apparent, but one may speculate that adrenal cortical hormone deficits or excesses may alter catechol amines in neurological tissues elsewhere, for example, the brain, in a similar or even more drastic fashion.

(2) Putative Relationship Between Autonomic and Adrenocortical Function in Mental Illness

The above findings concerning the dependence of adrenergic neurohumors on adrenocortical function may contribute to an understanding of the reported description of defective circulatory dynamics in chronic schizophrenics, as summarized by Hoskins (49). Such a view is concordant with Lewis's description of hypoplasia of the cardiovascular system in schizophrenia (49) and receives some support from the contrasting development of hypertension and in the vascular hyperplasia in hypercorticalism. The purported failure of the adrenal cortex to respond to appropriate forms of stimulation in schizophrenia (62) may be part of the same picture, though this area is still highly controversial (2).

This is but a partial picture and ignores central mechanisms in cardiovascular responses to pressor and depressor drugs. To this aspect the work of Funkenstein, Greenblatt, and Solomon (32, 33) has drawn attention and appears to supply a bridge between endocrinological thinking and concepts of autonomic nervous system functioning in mental disease. The two are not exclusive and may be reciprocally related and interdependent as the writer has pointed out (10). The connection between the work just summarized and that of Funkenstein *et al.* may be seen in the latter's finding that many schizophrenics exhibited defective pressor responses to epinephrine and a prolonged depressor response to mecholyl (Group V). One might conjecture that this is dependent on a relative inadequacy of adrenocortical secretion, since cases of Addison's disease exhibit a rise in blood pressure that is less than normal to epinephrine (8) and a prolonged depressor response to mecholyl (61).

If Funkenstein's results can be interpreted, at least in part, as indicating a difference in central responsiveness to the autonomic drugs used in his different categories of cases, it is highly likely that there is a change in the diencephalon, whatever may be going on in other parts of the brain. It is of interest that electroconvulsive therapy, which also stimulates adrenocortical secretion (40), restores the response of these drugs to normal (33). This is of interest to anyone familiar with the recent work of Shagass (78). This colleague of the writer has developed an index of cerebral and behavioral response to sodium amytal which he terms "sedation threshold". Essentially the sedation threshold represents the amount of amytal required to produce slurred speech and accompanying quantitative changes in EEG fast activity. He has found considerable variation in the sedation thresholds of various clinical psychiatric groups, for example, low thresholds in psychotic depressions and conversion hysterics, high thresholds in anxiety states and obsessionals. In non-psychotic patients the threshold seems to be a function of degree of anxiety and tension.

It would be of interest to know how results of this type of study would correlate with the Funkenstein test and with hormone excretion patterns.

In order to forestall the censure of the more quantitatively-minded amongst you at the circumstantiality of the data so far summarized, may I point out that a correlation of isolated evidence at something approaching the intuitive level can act as a pointer for the direction in which proof must be sought.

(3) Differential Release of Adrenergic Neurohumors

The hypothalamus is so organized that it functions as the head ganglion of the autonomic nervous system. For that reason, it is closely associated with the release of adrenergic mediators by sympathetic nerves and the adrenal medulla. Recent developments have lent greater significance to its role in the regulation of the secretion of the neurohumors, epinephrine, and norepinephrine. Apparently these may be liberated independently. An explanation of this phenomenon is that sympathetic nerves may be aroused to release their mediator, norepinephrine, without the concurrent secretion of epinephrine by the adrenal medulla. The experiments of Redgate and Gellhorn (67), on which this view is based, indicate that mild electrical stimulation of the hypothalamus led to excitation of sympathetic nerves releasing norepinephrine only, while stronger stimuli caused epinephrine secretion by the adrenal. Another explanation depends upon the fact that the adrenal medulla has recently been shown to secrete both norepinephrine and epinephrine, the former chiefly on initial stimulation and a preponderance of the latter as the stimulus is continued (24). Since different types of reflex stimulation in the cat gave different proportions of these adrenergic substances in the adrenal vein blood, von Euler and Folkow (24) hypothesized that the two medullary hormones could be selectively secreted. Evidence for this opinion was obtained by stimulating different areas in the hypothalamus. Stimulation of one area gave a preponderance of epinephrine and of another area nearby a preponderance of norepinephrine in the adrenal vein blood. They felt that their results imply specific hypothalamic representation for nerves supplying different norepinephrine and epinephrine-secreting cells in the adrenal medulla.

That a preponderance of norepinephrine or epinephrine may be secreted by humans in different emotional states has been suggested by Funkenstein, King, and Drollette (34) as a result of their studies completed before the data of von Euler and others became available. After their psychophysiological studies of stress in students, they concluded that those expressing anger directed outwards had a secretion predominantly of norepinephrine, while those showing anger directed inwards, or anxiety, secreted epinephrine in excess of norepinephrine. This work calls for more direct measurement of the adrenergic substances, preferably in blood, but at the time of writing there is no adequate chemical method for distinguishing the two unequivocally. Urine lends itself more readily to analysis, but for this type of study is definitely second choice.

The implication of these findings of Funkenstein et al., if confirmed, are considerable. The data lend themselves to the framing of many hypotheses and to the design of many experiments in the study of both psychosomatic and psychotic cases. This adds one more reason to think of the hypothalamus, the autonomic nervous system, and the endocrines as inseparable elements of a reverberating circuit in which still higher CNS functions, such as the so-called "visceral brain" of MacLean (57) participates. Moreover, the hypothalamus is not only an important focus for regulating sympathetic nerve and adrenal medullary function, but it seems to bear a chemical synonymity to those peripheral neurogenically-derived tissues, for it houses adrenergic neurohumors itself. There is a remarkably high concentration of norepinephrine in this tissue, according to Vogt (86). This is not an anomalous finding, for certain data indicates its functional significnace. It may play a part in evoking the secretion of LH for it does do this, at least when placed in the third ventricle (76). Epinephrine is similarly active (15, 59), and in certain experiments has been shown to lead to ACTH release as well (56). Norepinephrine is ineffective in this respect, but as my associate, Saffran (75) and his co-workers have shown, it may act as a cofactor with a hitherto unrecognized fraction of the neurohypophysis to constitute the principal neurohumoral agent in the release of ACTH.

Another amine which is found in the brain is serotonin (5-hydroxytrypt-amine), about which there will be more to say later.

B. Relation of Epinephrine-like Substances to Changes in Mood, Thought, and Perception

(1) Experimental Psychoses

It has been known for some time that epinephrine injections often produce feelings of anxiety in man, whereas similarly severe cardiovascular changes accompanying the action of norepinephrine do not (37). Substantial results of systematic inquiry in this area are lacking. However, the administration of certain substances chemically allied to epinephrine have yielded less equivocal and more dramatic alterations of mood, thought, and perception. The picture they produce has been called a "model psychosis" by Fischer (27) and others.

The first of these substances with which we are concerned today is mescaline. This is an alkaloid extracted from peyotl (Anhelonium lewinii) and has actually been known for over 50 years for its ability to produce schizophrenic-like symptoms. Its properties, described most recently in detail by Hoch (45), have been of considerable academic interest, and a fillip to public curiosity was provided by Aldous Huxley in his book "Doors of perception". There he gave an erudite account of the esoteric effects he experienced after taking the drug (50). Symptoms after ½ to 1 gm. include autonomic changes such as pallor or flushing, and dilated pupils, with tremor and in-co-ordination. Psychotic manifestations include vivid visual hallucinations, in which symbolizations and infantile wish-fulfilling fantasies dominate the person. Auditory

hallucinations also occur and various alterations in somatic sensations. Delusions, depersonalization, and misinterpretation of environmental situations occur. Depression or euphoria, anxiety and fear may develop, but the experience passes in 10 or 12 hr.

Interest in drugs which produce mental changes was accelerated by the introduction of a still more active agent of this nature, namely, d-lysergic acid diethylamide (LSD). The discovery that LSD in minute amounts produced schizophrenic-like reactions is a classic example of serendipity. The chemist, Hoffman, while working with it in 1943, was alert enough to associate the odd mental symptoms he developed with the handling of that substance. This permitted deliberate testing on subjects, which was reported by Stoll in 1947 (84). He described changes using LSD in doses as low as 20 to 30 gammas (γ), such as, visual hallucinations, vegetative and motor symptoms, changes in feeling, tone, and thought. He considered the picture a dience-phalosis.

Rinkel was the first to report on the effects of LSD on this continent, at the 1950 meeting of the American Psychiatric Association. His colleagues and his observations are summarized in subsequent published reports (20, 51, 69, 70). Briefly, they found that the LSD reaction showed some aspects of an exogenous toxic picture, but the simulation of simple schizophrenic and turmoil states was common, as were transient paranoid trends. Schizoaffective and manic-like states were also met. Disturbances of thought processes, perception, behavior, mood, and affect were encountered. Depersonalization was often described by the subjects and catatonic phenomena were seen. All subjects showed disturbances of the autonomic nervous system, such as salivation, nausea, urgency of micturition, pallor, flushing, sweating, gastrointestinal cramps, faintness, and giddiness. They speculated that the action of the drug was on the higher centers of the central nervous system, including the highest sympathetic.

These findings are in accord with those of Hoch *et al.* (45, 46) who were working at the same time in this field, though placing the emphasis on mescaline as the experimental tool. They noted some minor differences in the reactions to the two drugs which may, in part, be due to the difference in route of administration. Sexual material was produced frequently in schizophrenics taking mescaline, but not in normals. LSD has been also used for psychotherapeutic purposes (6).

A brief digression on the chemistry of mescaline is indicated because re-examination of its constitution stimulated a new concept. The structural formula had been known for 25 years, but this was of no interest to psychiatrists till Osmond and Smythies (60) pointed out in 1952 that it was not very different from epinephrine.

In the process of philosophizing with their chemically sophisticated associates, they came to the speculative hypothesis that methylation of the phenolic groups of norepinephrine could produce a substance with mescaline-like properties, though they pointed out it would have to be more active than

Fig. 1.

mescaline which must be given in a dose of $\frac{1}{2}$ to 1 gm. to produce mental deviations. Further, they emphasized the extraordinary activity of LSD which produces its deviant effects in doses as low as $\frac{1}{2}$ to 1 γ per kgm. On only a slightly more elaborate basis than indicated by this summary, Osmond and Smythies suggested that schizophrenia was due to specific metabolic disorder of the adrenals, producing a mescaline-like, distorted, epinephrine derivative.

Starting from this prospect of a brave, new world in schizophrenia research, these workers, in collaboration now with Hoffer (47), had a happy contact with empiricism which advanced their cause. Friends informed them of curious psychological consequences associated with the use of "pink adrenaline". This sounded like an hallucinogen, a word coined by them. The proximity of knowledgeable chemists suggested that adrenochrome might be an important constituent. This is a decomposition product of epinephrine, but has the indole nucleus in common with LSD and serotonin, the recently discovered amine found in the brain as well as other tissue (88). They felt that adrenochrome could be derived from epinephrine in the body and, under stress in the schizophrenic, might be so formed. This substance was therefore prepared and given intravenously to several subjects with dramatic psychological effects somewhat similar to those of mescaline and LSD (47). They reported feelings of increasing keenness associated with feelings of losing touch with people, of loss of significance, of potential danger to life (in a near accident), loss of sense of distance, ideas of reference, and colored hallucinations. It also produced EEG changes and inhibited aerobic and anaerobic metabolism of brain tissue in the Warburg apparatus. They point out that this is the first time that a substance which probably occurs in the body has been shown to be active in this particular way.

FIG. 2.

The inspirational approach of these workers has its place, but verification is another matter. Rinkel, Hyde, Solomon, and Hoagland (70) have contributed a summary of their substantial observations. They did not get the same results using the semicarbazone of adrenochrome that the Saskatchewan workers obtained with adrenochrome itself. However, as Rinkel et al. (70) have pointed out, a further degradation product of adrenochrome, namely, "adrenoxin", may have caused the reported mental symptoms in the experiments of Hoffer et al. They are not prepared, therefore, to dismiss this type of approach without further study. One finding of relevance to the present survey is that LSD was found to stimulate the pituitary adrenal axis and to render the adrenal somewhat unresponsive to later ACTH stimulation (70).

(2) The Antimetabolite Hypothesis for Hallucinogens

In seeking an explanation for the phenomena under discussion, it is necessary to refer to some more strictly chemical observations, and for orientation in this area I am indebted to my colleague, Dr. T. L. Sourkes (80).

There is a large body of chemical work which attests to the theory that compounds which bear close chemical resemblance to naturally-occurring physiologically active metabolites may interfere with the normal utilization or breakdown of essential metabolites, and hence with normal metabolism (87).

These chemical relatives of normal intermediates in physiological processes have been called "antimetabolites". Woolley and Shaw (88) have applied this concept to the blocking effect shown by LSD on the action of serotonin on smooth muscle, speculating that it might act similarly in the brain. They picture naturally-occurring mental disease as arising from a deficiency of serotonin, brought about by metabolic processes regulating its synthesis or destruction. As an alternative, an excessive accumulation might occur if amine oxidase were inhibited by an antimetabolite competing for the destructive functional site.

Further studies by Brodie et al. (79), on serotonin, have shown that it potentiates the hypnotic effect of hexobarbital and that this effect could be

Fig. 3

antagonized by LSD. Since LSD also opposed the additive hypnotic effect of hexobarbital and reserpine, these workers suggest that reserpine may act (to tranquilize) by releasing serotonin from body depots. Support for this view was obtained by demonstration of a fivefold increase in the urinary excretion of 5-hydroxyindolacetic acid, a major metabolite of serotonin, following the administration of large amounts of reserpine. One source of this is the intestine (65), and early results should indicate whether the same change occurs in the brain. Somewhat inadvertently, the name of reserpine, one of the recently-introduced and astonishing tranquilizing drugs, has crept into our deliberations. It would take us too far afield to consider its effects today, or that of the equally amazing and active, but different, agent, chlorpromazine; but for our purposes today it should be noted that the chemical formula of reserpine includes a portion with the indole ring, marking its resemblance to serotonin, and possibly qualifying it as an antimetabolite.

Still more recently, another blocking agent against LSD psychosis has been introduced. It is Frenquel (α -4-piperidyl benzhydrol hydrochloride) the gamma isomer of Meretran. It is said to be active in naturally-occurring hallucinatory states, but considerations of space preclude further elaboration here (25, 26).

The inhibiting effect of mescaline, LSD, and serotonin on cortical terminal synapses in the cat's brain have been recorded by Marrazzi and Hart (59) and useful speculation advanced as to the mechanisms by which hallucinogenic disturbances might take place. Serotonin is many times more potent than the others.

C. The Adrenal Cortex, Mood, and Mentation

The growth of a pharmacodynamic approach to psychiatry has been paralleled by developments in endocrinology which show that naturallyoccurring hormones may produce mild to severe changes in personality. A matter of fundamental interest is that the culpable agent is a steroid of the glucocorticoid variety. Earlier in this review, evidence for the interrelationship of adrenocortical steroids and epinephrine and its demethylated derivative has been examined. Now the place of steroids in artificially-produced and naturally-occurring psychoses will be related. The trend of the times is to incorporation of the more dramatic of scientific advances in the lay press, and a good example of this is the historical summary and case report of a man treated with cortisone in a recent issue of the New Yorker (74).

(1) Clinical Observations

The development of severe changes in mood and mentation accompanying the therapeutic use of cortisone and ACTH was a striking finding for which adequate explanation is not yet available (7, 13, 14, 30, 36, 38, 54, 55). Mood elevation to the point of mania, or depression, delusions, hallucinations, paranoid and catatonic states, and schizophrenic-like states have all been encountered. Two major explanations for the mood or other changes are that they are: (1) a response to relief of symptoms, and (2) a direct effect of the steroids on brain metabolism. There is no reason to believe that these hypotheses are mutually exclusive nor that one is of more importance at some times than others. Evidence arising from the observation of psychoses occurring in patients with Addison's disease who are receiving cortisone suggests a direct cerebral metabolic effect (12, 18), but the predilection to mental aberration is already high in these cases (9, 23, 84). Lidz (54, 55) found a much lower incidence of psychotic reactions in the cases that he studied, and the inference is that they received more psychiatrically oriented care than cases reported by some others, in which the incidence was higher. The question of dosage unfortunately still remains a factor whose importance is difficult to assess (55). Fleminger (28) suggests that cortisone is more apt to lead to mood elevation and ACTH to depression.

The conversion of what appeared to be a steroid-induced euphoria into depression by an adverse psychological situation was well illustrated in a case described by Fox and Gifford (30). The depression lifted and the euphoria returned when the patient ventilated his feelings and wept about his unhappy adversity. Such an instance illustrates the fallacy of a rigid "either/or" attitude towards "physiological vs. psychological" agencies in this area.

The findings with cortisone and ACTH have led to a re-examination of mental changes in Cushing's disease and allied states of hypercorticalism, and the amassing of considerable evidence favoring the thesis that an endogenous excess of 11-oxycorticoids may lead to cerebral dysfunction (13, 36, 85). It is significant that some of these cases show mental changes before alterations in bodily configuration become apparent (14), and that some have been reported to be restored to their preillness mental status by unilateral adrenalectomy (1).

The mental picture in hypercorticalism, whether spontaneous, as in Cushing's disease, or induced by cortisone and ACTH, is varied, and ranged from

manic through schizoaffective to catatonic states and depressions. The validity of making a biological distinction between manic-depressive psychoses and schizophrenia is thereby heartily questioned, at least as presented in this group of cases. Curiously enough, the psychopathological changes in "cortisone psychoses" may respond to electroconvulsive therapy. A single case report, of possibly considerable significance, describes recovery from a psychotic episode of a case of Addison's disease who was refractory to electroconvulsive therapy before, but responsive after, being given cortisone (19).

(2) Steroid Metabolism in the Psychoses

Evidence arising from two principal groups of biochemical investigators implies aberrations in steroid metabolism in the psychoses. The first of these, Pincus and Hoagland (62), following up the earlier work of Hoskins (49) at the Worcester Foundation has presented data indicating that schizophrenics are defective with respect to adrenocortical function. They found that many patients failed to show a normal adrenocortical stress response and that this was paralleled by a similar failure in 72% of tested cases to respond to injected ACTH. Confirmation of these findings, at least in part, has been commented on by Hoagland (43) but the results have also been vigorously attacked and their significance minimized by Altschule (2).

In an extension of the above studies, the Worcester group have described a different resting pattern in the urinary excretion of adrenocortical steroids, the patients putting out an excess of 17-ketosteroids and significantly less of corticoids than the controls. In a finer analysis of the steroid excreted, Hoagland (44) reports that Romanoff found one substance present in all schizophrenic samples and absent in normal, and another absent in schizophrenic, but present in normals (63). Psychotic subjects were also found to differ from controls with respect to the per cent increases in 17-ketosteroids, uric acid, sodium, and potassium following various stresses and injected ACTH, the patients being less responsive. The output of urinary phosphates, on the contrary, was considerably in excess of that of the controls, a finding not yet adequately explained.

Adrenal steroid excretion studies in psychotics have also been undertaken by Hemphill and Reiss (41, 68) of Bristol, England. They have, on the whole, preferred longitudinal studies on a few patients to the cross sectional approach used by Pincus and Hoagland on a larger hospital population. Reiss (68) has described bigger variations in the steroid excretion in his mental patients, and a different diurnal curve in many. His group have also found differential changes, a rise in β -hydroxy-17-ketosteroids occurring simultaneously with a decrease in corticoids, a state usually accompanied by depressions. This finding is the opposite of that reported by Rizzo, Fox, Thorn, and Laidlaw (71), who observed a cyclothymic patient over many months and whose hypomanic state was accompanied by a low corticoid excretion. Another allied type of study is that initiated by Gjessing and extended in recent years by Stokes, Gornall, and their colleagues at Toronto (39). These workers have

been able to show alterations in adrenal steroids in the urine at the time of the psychotic attacks, which was predictable from the previously recognized nitrogen excretion changes (39).

The testing of the hypothesis that aberrant steroidogenesis may produce psychosis can only be undertaken by those few laboratories especially fitted to make the study, but others may profit by their endeavors. There are ancillary leads from such work which indicate that by altering the line of attack it will not be necessary to wait till all the steroid answers are in before continuing physiological and biochemical investigations in mental illness. One such is the recent report of Stevenson, Metcalfe, and Hobbs (81), who have shown that catatonic and paranoid schizophrenics react differently to epinephrine and ACTH as judged by eosinophile counts. The latter appeared hypoactive and hyporeactive while the catatonics behaved more like normals. These findings may help explain conflicting reports in the literature on adrenocortical function in schizophrenia.

Some hint as to how steroid deficit, excess, or aberration may operate in producing mental disturbances is contained in a recent account of the collaborative research between Hoagland, the steroid specialist, and Rinkel and Hyde, the LSD experts (44). They found that the urinary excretion of phosphates in the LSD-treated normal person was reduced to the low level exhibited by schizophrenic subjects, and that ACTH injection caused an increased phosphate excretion in both groups, not seen in normal controls. They suggest that an endogenous derivative of epinephrine metabolism may be the agent acting in schizophrenic patients after the manner of action of LSD in normals, though how this is related to steroid metabolism, they do not suggest.

All of these findings will impinge on, alter, and I trust, improve our ideas of homeostasis, particularly as it applies to mental mechanisms (11, 14, 17, 22, 52, 53, 77).

Since I began my scientific life in physiology, I am partial to viewing human processes in that light. It is my hope, however, that such an approach will contribute substantially to a sound psychology, and render comprehensive certain clinical observations. In the long run, we have to use psychological and psychodynamic concepts in dealing with human mental aberrations, though it is to be hoped that these can be made more exact by an exploitation of approaches founded on the basic sciences. A quotation taken from another context may be appropriate. It has been said (3) that "The eventual goals of this work are to learn: whether psychodynamic constellations can be meaningfully correlated with different classes of biological data".

Scientific investigation is an art (5) and works with intuition as well as statistics. Some truths cannot be stated in the latter way, but are nonetheless important for that, and can be verified and systematized in other ways. The ancients tried to systematize their conceptualizations regarding the four humors in a way which we find quaint. Perhaps we will yet be able to systematize on a sounder basis. Though our formulations must for long be

tentative, they should be construed not as mockery of our ignorance but with the wit to deny the dangers of uncertainty, and to provide points of departure for our observations and experiments.

References

- 1. ALLEN, C. Brit. Med. J. 1: 123. 1929.
- ALTSCHULE, M. D. Bodily physiology in mental and emotional disorders. Grune & Stratton, Inc., New York. 1953.
- 3. APTER, N. S. Bull. Menninger Clin. 18: 154. 1954.
- Armstrong, C. W. J., Cleghorn, R. A., Fowler, J. L. A., and McVicar, G. C. J. Physiol. 96: 146. 1939.
- BEVERIDGE, M. I. B. The art of scientific investigation. W. W. Norton & Company, Inc., New York. 1951.
- 6. Bush, A. K. and Johnson, W. C. Diseases of Nervous System, 11: 241. 1950.
- 7. CLARK, L. D., BAUER, W., and COBB, S. New Engl. J. Med. 246: 205. 1952.
- 8. CLEGHORN, R. A. Unpublished observations. 1939.
- 9. CLEGHORN, R. A. Can. Med. Assoc. J. 65: 449, 1951.
- CLEGHORN, R. A. The problem of variation in base line data especially as this relates to the establishment of psychiatric categories. Second conference in psychosurgery, New York. Public Health Service Publ. No. 156: 22. 1952.

- CLEGHORN, R. A. Psychiat. Quart. 26: 1. 1952.
 CLEGHORN, R. A. Am. J. Psychiat. 108: 568, 1952.
 CLEGHORN, R. A. Alterations in psychological states by therapeutic increases of adrenal cortical hormones. Ciba foundation colloquia on endocrinology. Vol. 3. J. & A. Churchill, Ltd., London. 1952. p. 187.
- 14. CLEGHORN, R. A. The interplay between endocrine and psychological dysfunction: In Recent developments in psychosomatic medicine. Edited by E. D. Wittkower and R. A. Cleghorn. Sir Isaac Pitman & Sons, Ltd., London. J. B. Lippincott Company, Philadelphia. 1954.
- 15. CLEGHORN, R. A. Psychosomat. Med. 17: No. 5, 367-376. 1955.
- CLEGHORN, R. A., FOWLER, J. L. A., GREENWOOD, W. F., and CLARKE, A. P. W. Am. J. Physiol. 161: 21. 1950.
- 17. CLEGHORN, R. A. and GRAHAM, B. F. In Recent progress in hormone research. Vol. IV. Edited by G. Pincus. Academic Press, New York. 1949.
- 18. CLEGHORN, R. A. and PATTEE, C. J. J. Clin. Endocrinol. and Metabolism, 14: 344. 1954.
- 19. CUMMING, J. and KEES, K. Successful treatment of a case of depression in Addison's disease. Can. Med. Assoc. J. In press.
- 20. DeShon, J. J., Rinkel, M., and Solomon, H. C. Psychiat. Quart. 26: 33. 1952.
- DONHOFFER, C. and MACLEOD, J. J. R. Proc. Roy. Soc. (London), B, 110: 125, 141, 158. 1932.
- Engel, G. L. Homeostasis, behavioural adjustment and the concept of health and disease. In Mid-century psychiatry. Edited by R. R. Grinker. Charles C. Thomas, Publisher, Springfield, Ill. 1953. p. 33.
- 23. ENGEL, G. L. and MARGOLIN, S. G. Arch. Neurol. Psychiat. 45:881. 1941.
- 24. EULER, U. S. v. and FOLKOW, B. Arch. exptl. Pathol. Pharmakol. 219: 242. 1953.
- 25. Fabing, H. D. Science, 121:1. 1955.
- FABING, H. D. Neurology, 5: 319. 1955.
- 27. FISCHER, R. J. Mental Sci. 100: 623. 1954.
- 28. FLEMINGER, J. J. Mental Sci. 101: 123. 1955.
- FOLKOW, B. and EULER, U. S. v. Selective activation of nor-adrenaline and adrenaline producing cells in the suprarenal gland of the cat by hypothalamic stimulation. Circulation Research, 2: 191. 1954.
- 30. Fox, H. M. and Gifford, S. Psychosomat. Med. 15: 614. 1953.
- 31. FRITZ, I. and LEVINE, R. Am. J. Physiol. 165: 456. 1951.
- Funkenstein, D. H., Greenblatt, M., and Solomon, H. C. J. Nervous Mental Disease, 108: 409. 1945.

- 33. Funkenstein, D. H., Greenblatt, M., and Solomon, H. C. Psychosomat. Med. 14: 347. 1952.
- FUNKENSTEIN, D. H., KING, S. H., and DROLETTE, M. A study of the direction of anger during a laboratory stress-inducing situation. Psychosomat. Med. In press. The experimental evocation of stress. Symposium on stress. Army Medical Service Graduate School, Washington, D.C. 1953. p. 304.

GERARD, R. W. Science, 122: 225. 1955. Am. J. Psychiat. 1955.
 GLASER, G. H. Psychosomat. Med. 15: 280. 1953.

37. GOLDENBERG, M., ARANOW, H., JR., SMITH, A. A., and FABER, M. Arch. Internal Med. 86 : 823. 1950.

GOOLKER, P. and Schein, J. Psychosomat. Med. 15: 589. 1953.

GORNALL, A. C., EGLITIS, B., MILLER, A., STOKES, A. B., and DEWAN, J. G. Am. J. Psychiat. 109: 584. 1953.

40. GRAHAM, B. F. and CLEGHORN, R. A. J. Clin. Endocrinol. 11: 12. 1951.

- 41. HEMPHILL, R. E. and REISS, M. ACTH in psychiatry. Intern. Congr. Psychiat., Paris. 1950.
- 42. HERMAN, M. and NAGLER, S. H. J. Nervous Mental Disease, 120: 268. 1954.

43. HOAGLAND, H. Intern. Record of Med. 166: 183. 1953.

44. HOAGLAND, H., RINKEL, M., and HYDE, R. W. Arch. Neurol. Psychiat. 73:100. 1955.

45. Носн, Р. Н. Am. J. Psychiat. 107: 607. 1951.

46. Hoch, P. H., CATTELL, J. P. and PENNES, H. H. Am. J. Psychiat. 108: 579. 1952.

47. HOFFER, A., OSMOND, B., and SMYTHIES, J. J. Mental Sci. 100: 29. 1954.

48. HOKFELT, B. Acta Physiol. Scand. Suppl. 92, Vol. 25. 1951.

49. Hoskins, R. G. The biology of schizophrenia. W. W. Norton & Company, Inc., New York. 1946.

50. Huxley, A. The doors of perception. Harper & Brothers, New York. 1954.

- 51. HYDE, R. S. VON MERING, O., and MORIMOTO, K. J. Nervous Mental Disease, 118: 266. 1953.
- 52. Kubie, L. S. Psychosomat. Med. 10:15. 1948.

53. LEHMANN, H. E. Psychiatry, 15: 387. 1952.

54. LIDZ, T. Am. J. Psychiat. 108: 650. 1952.

- Lidz, T., Carter, J. D., Lewis, B. I., and Surrat, C. Psychosomat. Med. 14: 363. 1952.
- Long, C. N. H. Regulation of ACTH secretion. In Recent progress in hormone research.
 Vol. 7. Edited by G. Pincus. Academic Press, Inc., New York. 1952. p. 75.
 MacLean, P. D. Studies on limbic system ("Visceral Brain") and their bearing on
- psychosomatic problems. In Recent developments in psychosomatic medicine. Edited by E. D. Wittkower and R. A. Cleghorn. J. B. Lippincott Company, Phila-1954. delphia.
- 58. MARKEE, J. E., EVERETT, J. W., and SAWYER, C. H. The relationship of the nervous system to the release of gonadotrophin and the regulation of the sex cycle. progress in hormone research. Vol. 7. Edited by G. Pincus. Academic Press, Inc., New York. 1952. p. 193.
- 59. MARRAZZI, A. S. and HART, E. R. Science, 121: 365. 1955.
- 60. OSMOND, H. and SMYTHIES, J. J. Mental Sci. 98: 309. 1952.

61. PERERA, G. A. J. Am. Med. Assoc. 128: 1018. 1945.

62. PINCUS, G. and HOAGLAND, H. Am. J. Psychiat. 106: 641. 1950.

- PINCUS, G., HOAGLAND, H., FREEMAN, H., ELMADJIAN, F., and ROMANOFF, L. Psychosomat. Med. 11: 74. 1949.
- 64. PINCUS, G., SCHENKER, V., ELMADJIAN, F., and HOAGLAND, H. Psychosomat. Med. 11: 146. 1949.

65. PLETSCHER, A., SHORE, P. A., and BRODIE, B. B. Science, 122: 374. 1955.

66. RAMSEY, E. R., GOLDSTEIN, M. S., and LEVINE, R. Am. J. Physiol. 165: 450. 1951.

67. REDGATE, E. S. and GELLHORN, E. Am. J. Physiol. 174: 475. 1953.

68. REISS, M. Intern. Record of Med. 166: 196. 1953.

- 69. RINKEL, M., DE 572. 1952. DESHON, H. J., HYDE, R. W., and SOLOMON, H. C. Am. J. Psychiat. 108:
- 70. RINKEL, M., HYDE, R. W., SOLOMON, H. C., and HOAGLAND, H. Am. J. Psychiat. 111: 881. 1955.

- Rizzo, N. D., Fox, H. M., Thorn, G. W., and Laidlaw, J. C. Ann. Internal Med. (No. 4) 41: 798. 1954.
- 72. ROME, H. P. and BRACELAND, F. J. J. Am. Med. Assoc. 148: 27. 1952.
- 73. ROME, H. P. and BRACELAND, F. J. Am. J. Psychiat. 108: 650. 1952.
- 74. ROUSCHE, B. Annal of Medicine: Ten Feet Tall. The New Yorker. p. 47. Sept.
- 75. SAFFRAN, M., SCHALLY, A. V., and BENFEY, B. G. Endocrinology (No. 4), 57: 439-1955.
- SAWYER, C. H. In Discussion of Markee, Everett and Sawyer. Recent progress in hormone research. Vol. 7. Edited by G. Pincus. Academic Press, Inc., New York. 1952. p. 139.
- 77. SELYE, H. Stress. Acta Inc., Montreal. 1950.
- 78. SHAGASS, C. Electroencephalog. and Clin. Neurophysiol. 6: 22-233. 1954.
- 79. SHORE, P. A., SILVER, S. L., and BRODIE, B. B. Science, 122: 284. 1955.
- SOURKES, T. L. The concept of metabolic blocks in psychiatric research. Psychopharmacological Conference, March, 1955. Allan Memorial Institute, Montreal, Canada.
- 81. STEVENSON, J. A. F., METCALF, E. V., and HOBBS, G. E. Arch. Neurol. Psychiat. 70: 802. 1953.
- VENSON, L. History of Psychopharmacology. Psychopharmacological Conference, March, 1955. Allan Memorial Institute, Montreal, Canada. 82. Stevenson, L.
- STOLL, W. A. Schweiz. Arch. Neurol. Psychiat. 60: 279. 1947.
 STOLL, W. A. Die psychiatrie des morbus Addison. G. T. Verlag, Stuttgart, 1953.
- 85. TRETHOWAN, W. H. and COBB, S. Arch. Neurol. Psychiat. 67: 283. 1952.
- 86. Vogт, M. J. Physiol. 123: 451. 1954.
- 87. WOOLLEY, D. W. A study of antimetabolites. John Wiley & Sons, Inc., New York. 1952.
- 88. Woolley, D. W. and Shaw, E. Brit. Med. J. 2:122. 1954.

DISCUSSION: J. K. W. FERGUSON¹

As pointed out by Dr. Cleghorn there has been an enormous resurgence of interest in the effects of hormones and drugs on mentation and emotion. Many new drugs have been introduced for their effects on the psyche. Many other substances have been studied for their possible significance in the production of mental symptoms. Conflicting claims emphasize the laxity of experimental methods used. Attention is drawn to the recent work of Dr. H. K. Beeker (1) on assessing the subjective effects of drugs. He states clearly and emphatically the conditions necessary for valid experimentation in this field. Most workers in the field could study his considered opinions with profit.

^{1.} BEEKER, H. K. J. Am. Med. Assoc. 158: 399. 1955.

¹Contribution from the Connaught Laboratories and Department of Pharmacology, University of Toronto, Toronto, Ontario.

COMMONWEALTH BUREAU OF ANIMAL NUTRITION

ROWETT RESEARCH INSTITUTE, BUCKSBURN, ABERDEENSHIRE, SCOTLAND

Publications and Services

NUTRITION ABSTRACTS AND REVIEWS

World literature on the nutrition of man and animals is abstracted in sections embracing methods of analysis and experimental techniques; composition, digestion, and utilization of food and systems of feeding; growth, reproduction, and all physiological processes concerned with the use of food; the relation of diet to health and disease; the feeding of livestock.

Each number contains also a Review Article dealing with some subject of current practical importance.

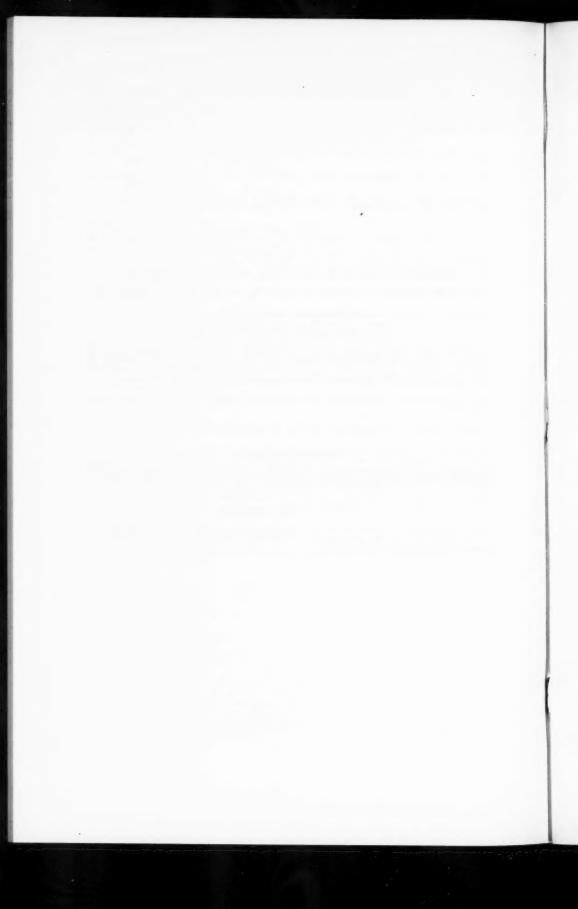
Published quarterly. Price 105s. per volume. The current volume is Vol. 26.

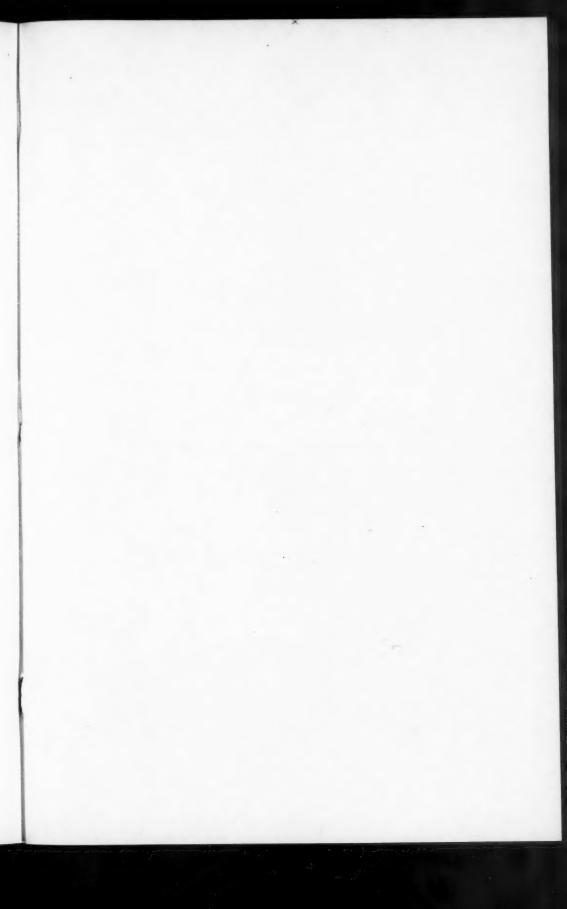
TECHNICAL REPORTS

From time to time the Bureau prepares Technical Communications on subjects of practical importance. A list of these may be had on request.

CONSULTATION SERVICE

The Bureau is prepared, within the limits of its information, to answer enquiries from workers in laboratory research, field investigations, or nutritional planning, who do not themselves have the necessary access to literature.







NOTES TO CONTRIBUTORS

Canadian Journal of Biochemistry and Physiology

MANUSCRIPTS

General.—Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½×11 in. The original and one copy are to be submitted. Tables, and captions for the figures, should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered. Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Greek letters or unusual signs should be written plainly and explained by marginal notes. Superscripts and subscripts must be legible and carefully placed. Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

Abstract.—An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

References.—These should be designated in the text by a key number and listed at the end of the paper, with the number, in the order in which they are cited. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given, and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent List of Periodicals Abstracted by Chemical Abstracts. All citations should be checked with the original articles.

Tables.—Tables should be numbered in roman numerals, and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should not be used. Numerous small tables should be avoided.

ILLUSTRATIONS

General.—All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations.

Line drawings.—Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines must be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots must be solid black circles large enough to be retained when reduced. Letters and numerals should be neatly made, preferably with a stencil (do NOT use typewriting), and be of such size that the smallest lettering will be not less than 1 mm high when the figure is reduced to a suitable size. Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. Wherever possible two or more drawings should be grouped to reduce the number of cuts required. In such groups of drawings, or in large drawings, full use of the space available should be made; the ratio of height to width should conform to that of a journal page (4½×7½ in.), but allowance must be made for the captions. The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

Photographs.—Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard, with no space between those arranged in groups. In mounting, full use of the space available should be made. Photographs are to be submitted in duplicate; the duplicate set of grouped photographs should be unmounted.

REPRINTS

A total of 100 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased at the time of publication. Charges are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, $81/2 \times 11$ in.) and including the space occupied by illustrations. Prices and instructions for ordering reprints are sent out with the galley proof.

Contents

	Page
The Metabolism of the Erythrocyte. X. The Inorganic Pyrophosphatase of the Erythrocyte—A. Malkin and O. F. Densledt The Metabolism of the Erythrocyte. XI. Synthesis of Diphosphopyridine	121
The Metabolism of the Erythrocyte. XI. Synthesis of Diphosphopyridine Nucleotide in the Erythrocyte—A. Malkin and O. F. Denstedt	130
The Metabolism of the Erythrocyte. XII. Diphosphopyridine Nucleotide Nucleosidase of the Rabbit Erythrocyte—A. Malkin and O. F. Densledt	141
Studies on Human Adrenal Steroids. 1. The Effect of Corticotropin on Components of the Free and Conjugated Plasma C ₂₁ Adrenal Steroid Fractions—C. M. Southcott, S. K. Gandossi, A. D. Barker, H. E. Bandy, Hamish	141
McIntosh, and Marvin Darrach- "Reflex" Anuria in the Dog-Sydney M. Friedman, Roland W. Radcliffe, J. E. H.	146
Turpin, and Constance L. Friedman Effect of Cortisone on the Intracellular Distribution of Phosphatases and Ribonucleases in Rat Liver—Claude Allard, Gaston de Lamirande, George Weber,	158
and Antonio Cantero	170
Ramachandran The Constituents of Cod Liver with Vitamin B ₁₂ Activity for Lactobacillus	180
leichmannii—Beryl Truscott and P. L. Hoogland The Role of A-esterase in the Acute Toxicity of Paraoxon, TEPP, and	191
Parathion—A. R. Main The Effect of Chlorpromazine Given with Reinfusion on the Mortality Rate	197
from Standardized Hemorrhagic Shock in the Rat—G. F. Carruthers and C. W. Gowdey	217
The Metabolism of the Erythrocyte. XIII. Enzyme Activity in the Reticulocyte—D. Rubinstein, P. Ottolenghi, and O. F. Denstedt	222
Spectrophotometric Analysis of Proteins by a Selective Filter Technique— E. Annau	236
The Gastric Endocrine and Exocrine Response to Histamine in Dogs and Effect of Passage of Blood Through the Gastric and Hepatic Vessels on its Pepsinogen Content—K. Kowalewski, S. T. Norvell, Jr., and Walter C. MacKenzie The Relative Potency of Adrenal Corticoids by the Thymus Involution	
Method—N. R. Stephenson	253
Lipid and Water Levels in the Kidneys of Albino Rats Bearing Walker Carcinoma 256—Eldon M. Boyd and Arne O. Tikkala The Degradation of Desoxyribonucleic Acid During Alkaline Hydrolysis—	259
R. O. Hurst - Electrophoresis of Autoprothrombin and Biothrombin—Walter H. Seegers,	265
Takeshi Abe, and Richard L. Fenichel Comparative Biochemical Studies on Normal and on Poliomyelitis Infected	270
Tissue Cultures. I. Observations on Synthetic Nutrient Mixtures Incu- bated with Tissue Cultures of Normal Kidney—Ernest Kovacs	273
SYMPOSIUM ON THE CHEMISTRY AND PHYSIOLOGY OF PHOSPHOLIPIDS	
The Synthesis of Glycerolphosphatides—E. Baer	288
Discussion: C. S. McArthur - The Chemistry of the Phosphoinositides—J. Folch and F. N. LeBaron -	302 305
Discussion: C. C. Lucas	318
Chemistry of the Sphingolipides-H. E. Carter, Demetrius S. Galanos, and	
Y. Fujino	320
Discussion: J. F. Berry	331
The Biological Synthesis of Phospholipids—Eugene P. Kennedy -	334
Discussion: G. C. Buller Metabolism of Phospholipids in vitro—L. E. Hokin and Mabel R. Hokin	347 349
Discussion: R. J. Rossiler	358
The Function of Phospholipids-J. M. R. Beveridge	361
Discussion: Oroille F. Denstedt	370
SYMPOSIUM ON NEUROPHYSIOLOGY	
The Structural Basis of Some Corticodiencephalic Relations - J. Auer	372
Discussion: M. L. Barr	379
The Electrophysiological Approach to the Problem of Learning-	
B. Delisle Burns	380
Discussion: J. W. Pearce Pharmacological and Endocrinological Influences on Mentation and	388
Emotions—R. A. Cleghorn	390
Discussion: J. K. W. Ferguson	404

